

Getting scientists interested in policy

SCIENCE policy begins to bite—that is a clear message emerging from the annual reports of many bodies supporting science, and most recently from the Science Research Council (SRC), guardian of £70 million of British research from neurobiology to high energy physics. The message comes equally from the international organisations.

For years the last thing that most practising scientists wanted to know about was the machinery by which policy for science was evolved. After all the money generally could be relied on to roll in provided the proposal sounded plausible enough and dreary matters of policy could be left to those amiable and harmless civil servants in London, reinforced with sensible and central professors prepared to commute to endless committee meetings. We neither knew nor cared who they were, what they did in our name or how we were represented in international organisations because the funding was sufficiently pluralistic that if A regretfully couldn't provide, B probably would. And we were mercifully spared from Five Year Plans and the like, which we knew stultified Soviet science, for example. Even when a little forecasting was done and there was talk of shared facilities, we knew that if we produced the goods we would be allowed to go our own way.

The first shock came, of course, in the very late 1960s when university expansion ended and the devaluation of sterling was indeed starting to get at the pound in our pockets. But the real blows have come within the past two years when the budgets for science has been unable to keep up either with rising costs or with the ever increasing demands that scientists make on it. Added to this, universities find themselves with insufficient funds to guarantee that vacant posts can be filled. So the words priorities and planning will assume a greater significance and scientists will find themselves increasingly subject to decisions made for them by councils and committees. The words in the SRC report "... the Council have provisionally concluded ... that the priorities for astronomy, engineering and the area supported by the Science Board should be sharpened at the cost of reductions in other expensive programmes ..." can only be a foretaste of what is to come in straitened times.

It is impossible to argue with this in principle. If the government pays, then the government has the right to assert priorities. Moreover in some respects, particularly at the moment as regards questions of manpower, one could wish for more central direction. Do we really want to see, in the absence of some corrective pressure in schools and universities, a continuation of such trends as those which lead to many astronomers and few chemists? Nevertheless the very lack of prior interest in science

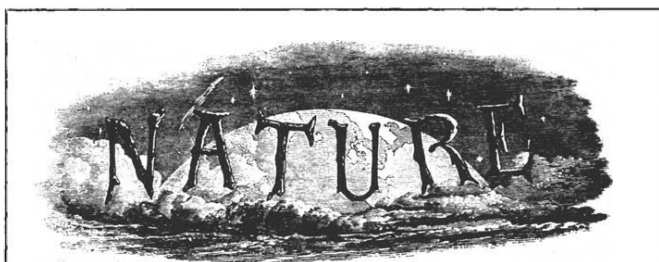
policy among most scientists raises some important questions.

To what extent are those who make and implement policy responsible to the scientist whose livelihood may be threatened? The short answer is not at all, neither in terms of being elected by him nor in terms of being required to discuss decisions with him. This is hardly to say that if you call up, say, the SRC and ask for extension you will not be able to speak with the chairman. You can, and he doubtless will give a civil reply. Mechanisms do not exist, however, for ensuring that the grass roots has any say whatever in the appointment of those in authority or who represent the United Kingdom internationally. No more do they exist for requiring that decisions be open to scrutiny, criticism and maybe modification in the light of this criticism.

It is not obvious that the system by which scientists are appointed rather than elected should be changed. Much dedicated work is done by people who would have no stomach for an electoral process and in the absence of party labels most electors confronted with a list of names about which they know nothing will vote at random—and in any case, who votes? But one would have greater confidence that an election was unnecessary if there were more frequent occasions on which rank-and-file scientists could listen to their representatives explaining decisions and listening to opinions. This is partly the function of the scientific press but mainly it needs some regular forum where scientists can get together.

This would be an ideal function for the British Association (BA) to perform. We have already proposed that the BA should take a more active role in making scientists more aware of their common interests by the forming of local cells, and one of the most valuable things that such cells could do would be to generate an enhanced interest in the questions of decision-making on scientific matters at governmental level. Such regular opportunities to talk about the problems at the top could hardly be other than most welcome to those who have, in the name of scientists, to try and share out a diminishing cake.

A hundred years ago



THE commotion created in the Paris School of Medicine by the false rumour spread by the *Figaro* has been beyond bounds; not only was M. Wurtz, the Dean, cheered, but M. Chauffard, one of the professors belonging to the clerical party, was hooted, and unable to deliver his lecture. The disorder having been renewed in spite of all precautions taken by M. Wurtz, the School of Medicine has been closed for a month. If students again exhibit a riotous spirit, the ringleaders will be prosecuted before a Council of War; which is a lawful proceeding, Paris being placed under a state of siege.

From *Nature*, 11, 56, November 19, 1874



Weather warning: you are now experiencing a climatic change

John Gribbin has recently been visiting climatic research centres in the United States and Canada as part of a Glaxo Travelling Fellowship. In this article he discusses work now in progress, the hopes of some of the scientists active in the field, and the pressing need for more research in view of world food problems.

THE name of Reid Bryson, Director of the Institute for Environmental Studies (IES) at the University of Wisconsin-Madison, is one which arouses strong response from most climatologists and meteorologists. His outspoken views on the imminence of global catastrophe and on the form of food shortages, and the degree to which he blames man's activities for the decline in global temperature over the past two decades, are either taken at face value or fairly strongly opposed—there is no middle ground.

Bryson and his group are particularly concerned with two aspects of the changing climate: determining precise quantitative parameters of past climates from analysis of pollen samples and predicting the development of present climatic trends, with special reference to the problems of food production. The former line of study causes no breast beating even among Bryson's most vociferous opponents. In its latest form, this study involves analysing pollen from the sediment layers (varves) in cores obtained from lakes in the north-eastern United States. The thickness of the varves, laid down in successive years, provide a guide to the year-by-year changes in mean climate; but that is at best a crude measure and the application of pollen analysis to the cores provides potentially the best indicator yet of how past climates have changed.

The continuous varve sample goes back for some 9,000 years—a longer and more complete record than the tree rings which have already proved so useful to climatologists. They provide more information over the whole span and there is the additional bonus of inorganic material from the sediments which provides other information about age and climate. So far, the Madison team has established a climatic

chronology made up of one sample per decade over the 9,000-years; work is now in progress on more detailed studies.

The facts which emerge from the analysis of past climates often encourage dramatic presentation. Bryson mentions "sharp" changes occurring within 17 years which are revealed by pollen studies. Meteorologists have argued that the atmosphere simply cannot change dramatically in such a short time, but, as Bryson puts it, "the theories must be made to fit the facts, not the other way around". And the facts he presents certainly require urgent explanation, suggesting as they do that it is possible to go from an interglacial regime (such as the present situation) to full glacial conditions in only 100 years.

According to Bryson, man's activities as an atmospheric polluter have reached a stage where that may be exactly what we are in for. Like many other climatologists, he sees volcanic activity as a prime cause of past changes, because of the effect of dust on the transmissivity of the atmosphere. Unlike some of his contemporaries, however, Bryson is convinced that man-made 'dust' is now producing a cooling trend as significant as any recorded effect of volcanic dust. In his own words "the troposphere shows clear evidence of a man-made effect since the 1940s", and this corresponds to the global cooling which has been going on since about that time. Bryson rejects the suggestion that variation in the Sun's activity could be responsible for a major part of this trend. "Don't tell me this is a major effect—how could it be physically?" was his response to my suggestion that the possibility deserved further study. This response was not entirely convincing, however, in the light of his own remarks about the need for theories to fit the facts, rather than the reverse.

Still, even if he does not go along with this particular controversial idea Bryson has plenty of publicity-worthy ideas of his own. Some of the latest work to emerge from the IES points to a relation between the incidence of condensation trails produced by jet aircraft over the North Atlantic, loss of sunlight and reduction in the euphotic zone of the oceans, a reduction in nutrients, and a delay in warming, which produces less plankton at a later time in the year than would

otherwise be the case. This delayed and decreased bloom of zooplankton is just what has been observed since 1950, selectively under the jet route, according to Bryson's figures.

But in spite of the 'gee whizzery' of such attempts to arouse public interest in the problem of climatic change there is an underlying seriousness to this work which justifies all the publicity-grabbing efforts. Bryson and his colleagues have produced projections of the fall in world food reserves which ought to be enough to shake even the dullest politician. By 1975, we could reach a situation in which only 8 to 10 days' supply of food remains in reserve—and the food distribution 'pipeline' needs 8 days' supply to keep things moving as they are at present. This bears out calculations by the World Meteorological Organisation (see *Nature*, **250**, 176; 1974) which highlighted the fact that there are no longer any substantial reserves of either food or unused farming land available. And it is his efforts to publicise this situation which are seen by other climatologists as Bryson's chief contribution to the problem of tackling present climatic change.

There is certainly a growing public awareness in North America of the fact that something is going wrong with the weather. The driver of the airline bus in Madison commented on the early frosts which have affected local farmers drastically, and a TV weather forecaster on a Chicago station commented in passing on the "three early cold waves which haven't done much good for the food situation". Stephen Schneider, of the National Center for Atmospheric Research (NCAR) in Boulder agrees that the food situation is worrying, but stresses the sense of urgency conveyed by Bryson's work without echoing his sense of certainty.

According to Schneider, we do not know enough about the natural processes of climatic change, or about man's influence, to be dogmatic. As Francis Bretherton, Director of NCAR put it, climatic research has been the "Cinderella of the meteorological sciences for the past two decades"; at present we are still at the stage of planning and getting a programme together. But unfortunately the deterioration in climate, if it is real, will not wait for the necessary research to be carried out. Schneider sees the possibility of a severe famine situation in

*Reid Bryson of IES: outspoken
views on imminent global
catastrophe.*

Ethiopia this winter as just one example of the immediate problems to be faced.

For the past 10 to 15 years the world climate has generally been good for food production, but bad husbandry and lack of foresight has produced a crisis situation. Supply and demand are now too close for comfort; Schneider believes that even three years ago it would have been possible with good husbandry to provide for all except the most extreme weather conditions, but now we need good weather for several years simply to get back to a marginal position of safety. The problem, he says, is man-made—but in the sense of being man-made foolishness, not technological.

In the long term the situation still looks bleak to Schneider. Although the problems to be solved are within the abilities of modern technology (in terms of increased yield per acre, improved standards of living and simply better 'housekeeping') the kind of budget and effort required would rank with the present military budget of the United States. In those terms, the problems look insurmountable simply in a political sense.

In spite of this gloomy prognosis, workers at NCAR and elsewhere continue to tackle many aspects of the changing climate situation with an increasing level of activity. If we can survive the immediate crisis which has been produced as a result of the interaction of population, food supply and climate, then we will be much better armed to tackle similar problems in future. Studies such as that by Warren Washington (NCAR) of the importance of waste heat from power stations and an investigation by Canadian meteorologists into the effect of reservoirs on precipitation are typical of the kind of problems which could, and should, have been tackled ten or more years ago and which are essential if the world is to continue to support anything like the present level of population.

The Canadians—notably the Meteorological Applications Branch of Environment Canada—have an enviable reputation for foresight because they possess equations relating wheat yield to the weather in Canada in any particular year. This, however, is more an accident of history than anything else, as the Branch's Director, Gordon Mackay, is quick to point out. The entire economy of Canada is so dep-



endent on wheat that this problem was tackled from an economic viewpoint, not through any concern about world food supplies. Nevertheless, the result is that the Canadians do have an existing working technique for determining wheat yields, and this could well be adapted to forecasting crop variations in other parts of the world.

Mackay is adamant that with the limited staff and resources at his disposal the Meteorological Applications Branch must consider primarily problems which affect Canada, although, of course, he recognises that this requires some understanding of the circulation of at least the Northern Hemisphere. Indeed, Mackay says that meteorologists in Canada would not really be aware of the changing climatic situation without the speed of modern communications; in parochial terms, they have no real problem as yet, although farmers have begun to switch to crops which require a shorter growing season, to such an extent that some kinds of seed grain are now very difficult to find.

This, perhaps, points to one immediate way in which the twin problems of food and climate can be tackled even before a full understanding of climatic change is developed. Farmers around the world must give up their traditional crops if these are not producing good yields and turn to the kinds of crops which can be grown satisfactorily in the conditions now prevailing. Echoing the sentiments expressed by Schneider, Mackay said that 50% of the world food supply is wasted by bad management or des-

troyed by pests and disease, some of these, such as rust, themselves being related to climatic factors.

The outlook is certainly gloomy, unless governments do a lot more than just talk about the problems at such gatherings as the World Food Conference. And it is no use for the holders of the purse strings to expect that a sudden change of heart on their part will belatedly lead to dramatic new revelations about how climate is changing and what influence this is likely to have on food supply. As Mackay put it, the situation today in climatic research is not unlike the situation in meteorology a hundred years ago. At that time, the Canadian Meteorological Service was founded to provide storm warnings, and functioned at first more or less on the principle of hoisting a warning to tell shipping "you are now in the middle of a storm"; today, the descendants of that service can tell us "you are now experiencing a climatic change". How big that change is, which direction it is moving in and just what effect it will have on the human population of our planet are all questions which can be answered quickly only if climatic research is far greater priority than it has received so far. The overall impression gained from talking with those active in these studies today is that the effort needed is comparable only to the effort of a full scale war, and without this effort then a major 'crunch', in the form of famine, disease, war or some combination of the three, will inevitably reduce the world population drastically within a few years. □

international news

WHEN New Delhi was chosen three years ago as the site for the 26th International Congress of Physiological Sciences, the Indian government gave assurances that nobody would be prevented from attending on the basis of race, citizenship, religion, political philosophy, language or sex. But when the event took place last month, visas were denied to scientists from Taiwan, Portugal, Rhodesia and South Africa, and scientists from Israel were only permitted to attend after attempts to exclude them drew strong international protests.

Those events, which are of course commonplace for conferences held in the Soviet Union, have been brought to the attention of at least one influential international scientific body, with the result that India is likely to slip a few places in the list of desirable locations for scientific jamborees.

New Delhi was selected as the site for this year's meeting by the General Assembly of the International Union of Physiological Sciences (IUPS) in 1971 but it was not until this summer that it became known that the Indian government intended to deny visas to scientists from five countries with whose policies it disagreed. Although no official

India barred conference delegates

reasons have been given, it is generally assumed that the Israelis were to have been excluded because India wanted to stay on the right side of Arab oil suppliers, the Taiwanese because of Taiwan's expulsion from the United Nations, the Portuguese because of Portugal's colonial policies in Africa, and the Rhodesians and South Africans for obvious reasons.

When the news reached the United States National Committee for the IUPS, it immediately called the matter to the attention of the International Council of Scientific Unions (ICSU), a major international scientific body, and asked the Foreign Secretary of the National Academy of Sciences to open negotiations with the Indian Embassy in Washington.

At the same time, a delegation of physiologists in India met with the Prime Minister, Indira Gandhi, to try

to persuade her to allow free access to the meeting.

An ICSU General Assembly, held in Istanbul a month before the IUPS Congress, passed a resolution to the effect that repeated incidences of restrictions on free communication between scientists would result in the ICSU advice to its constituent unions not to plan scientific meetings in such countries.

As a result of those pressures, the Indian government relented a little by granting Israeli scientists 21-day landing permits, but it held fast on its decision to exclude Taiwanese, Portuguese, Rhodesian and South African participants. One factor in the decision to allow Israeli participation was probably the fact that three symposia associated with the congress were being held in Israel.

It turned out, however, that no Rhodesians or Portuguese were registered to attend and that the one South African who applied held a British passport. (He later elected to stay away when his South African wife was refused a visa, however.) But five Taiwanese who wanted to attend the meeting were refused visas and thus were excluded from the conference. □

INDIA'S Fuel Policy Committee (FPC, set up in 1971 in the Planning Commission to project a long term energy picture for the country) has finally submitted its report to the government. The Arab-Israeli war of October last year had apparently caught the FPC napping; it was all set to submit its report in December 1973 but found to its chagrin a good number of its assumptions and calculations demolished by a series of unprecedented rises in oil prices. It took almost a year for the FPC to surface again with a report.

One of the central recommendations would like to see the country design her energy policy for the next few decades on the assumption that coal rather than oil would be the energy base of India's economy.

The committee's forecast of India's energy needs by the end of the Fifth Plan period (1978-79) is as follows: 135 million tonnes (Mt) of coal; 34.4 Mt of mineral oil and petroleum products; 120 billion kW h of electricity and 123 Mt of firewood, charcoal and dungcake.

To give effect to a coherent energy

policy, the FPC has made suggestions for administrative reorganisation; it would like the government to set up an energy commission and also an

Indian power plan

from Narender K. Sehgal

energy board comprising representatives from the Planning Commission and ministers concerned with petroleum, mines, railways and irrigation.

In line with its 'back-to-coal' recommendation, the committee has suggested that all new fertiliser projects should plan to use coal as feedstock. But it has also urged that priority should be given to exploration for oil and uranium.

The FPC has strongly recommended popularisation of gobar (dung) gas plants for more effective utilisation of this resource in the domestic and rural sectors. (Incidentally, gobar gas had not found any mention in the FPC's earlier energy projection which it was about to submit in December 1973).

For power generation, the FPC has suggested that hydroelectric schemes on

river systems to the extent of 80 to 100 million kW capacity, be investigated and installed within the next 20 to 30 years. It would also like power stations to be set up at coal pithead sites to ease pressure on movement of coal to existing distant thermal stations.

With regard to nuclear power, the FPC expects that by 1985-90 the country's uranium resources will be able to support an installed capacity of 6 to 8 lakh MW for three decades, and that by that time fast breeder reactors too will have come on line to turn thorium (which India has in great abundance) into fissionable fuel.

The FPC is not enthusiastic about installation of 'captive' power stations by industries (private or public) because, in its view, that would not be in the "overall national interest".

On energy research and development, work on conservation of coal should be given urgent attention since, in the committee's view, reserves of coking coal are not expected to last much longer than 40 years or so, whereas because of the expected industrial growth coal requirements will increase five-fold within the next 20 years.

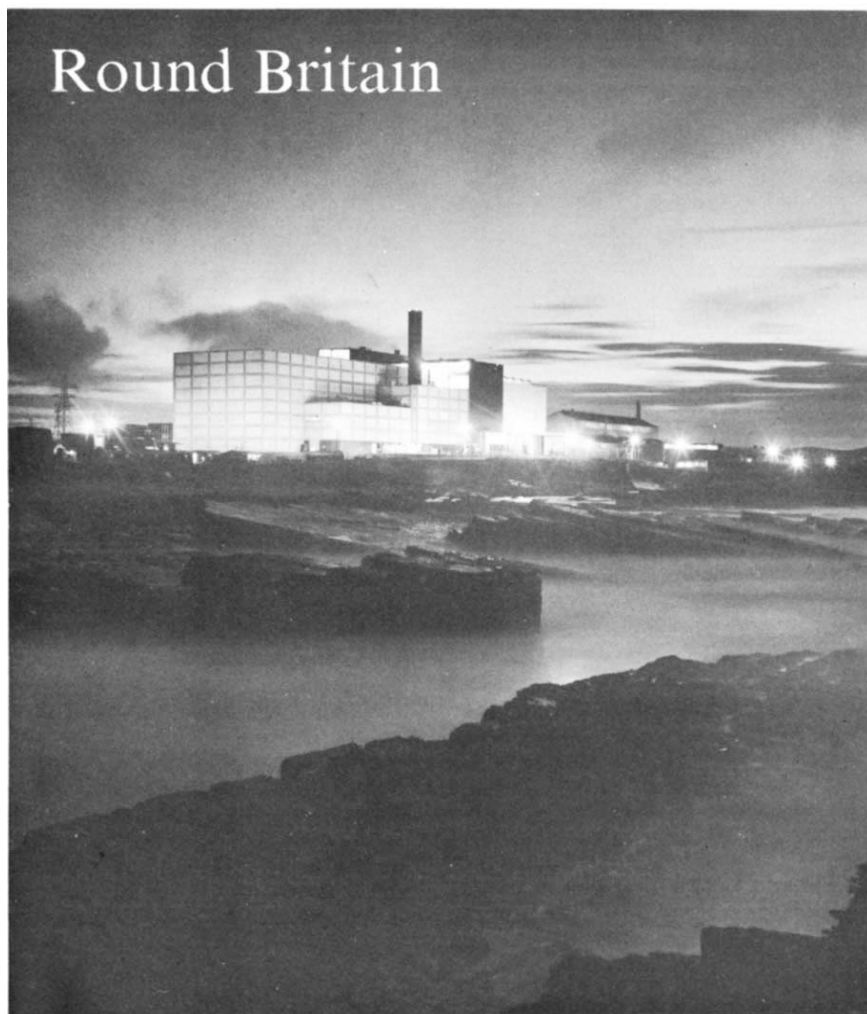
IN view of the emergency cuts in public spending last December, the Science Research Council (SRC) will have in real terms 2% less money to spend in the next year, but promises in its annual report that direct grant support to the universities will be maintained at the present level—reassuring news in the present financial crisis. The real cut in expenditure rather than the planned increase of $1\frac{1}{2}\%$ will mean, however, that the council has to defer a number of major projects. A large slice of the SRC's budget (about one-third) goes on international projects such as ESRO, CERN and the reactor at the Institut Lane-Langevin, and fluctuating exchange rates over the past year have inflated the real contribution to international programmes.

The probable cuts in university expenditure on research loom large in the SRC's thinking for the future. The cut-back in university spending is likely to throw more of the burden of supporting basic research on to the SRC at a time when their funds are not increasing.

This will mean that the SRC will have to be much more selective about new projects to be supported. Provisionally, the council has decided that the growth of postgraduate studentships should not be more than 1% a year and that astronomy, engineering and the activities covered by the Science Board (genetics, enzyme technology, neurobiology and various branches of physics and chemistry which do not come under the other boards) should be given priority at the expense of some other programmes. The establishments run by the SRC will also have to face a period of restraint to help raise resources for new facilities which only the council can provide.

- The Prototype Fast Reactor at Dounreay in Scotland (above) is producing some 40 MW of heat but is not expected to feed electricity into the grid for a few weeks yet. That event will be the culmination of more than eight months of testing since the reactor went critical in March. The eight months have not been without their problems, however, even though these have proved to be relatively minor—for example, a tiny leakage of steam into one of the secondary sodium circuits and blockage of the seawater input to the steam condensers by seaweed.

The United Kingdom Atomic Energy Authority (UKAEA) is, however, looking much further ahead than the time when the PFR generates its designed 250 MW of electricity. The first commercial fast reactor (CFR1) should,



according to Mr R. V. Moore, Managing Director of the UKAEA Reactor Group, be started in 1977 and completed in 1983. The present design envisages an output of 1,300 MW but an operating temperature of 500°C (50°C lower than that of the PFR) which would widen the choice of possible materials for its construction.

The big question is whether CFR1, assuming it gets the green light, will be built in as remote a corner of the country as Dounreay. Mr Moore thinks not.

- "The married are viewed sympathetically." With those ambiguous words a fellow of Wolfson College led the way into the family accommodation of Oxford's newest college, conceived eight years ago as a means of offering a home to two of the less-well catered-for groups—graduate students and university staff entitled to but not possessing fellowships. At that time, says Sir Isaiah Berlin, the President of Wolfson, the university was faced with the threat either of "wholesale migration to the western hemisphere, or worse still, blocking legislation in the

Congregation". Scientists predominate, reflecting not only their preponderance amongst graduate students but also the many uncatered-for scientific staff around Oxford. Now they seem regally looked after; through £3.2 million of Wolfson and Ford money in a building erected on the site of J. S. Haldane's house by the river.

Even if the senior members lack one or two perks such as a High Table, the environment in the coeducational college is idyllic by many standards and the recognition afforded to wives and families almost unique in Oxbridge. Down by the river, in the specially built loop for parking punts, the economic crisis seemed a thousand light years away. But can Wolfson retain its originality? Obviously the initial nucleus of fellows and the first generation of graduate students has a crusading zeal and is united by, if nothing else, a sense of coming together to improve the lot of those whom the university has left out. Whether this feeling can continue depends on the college's continuing ability to identify and support people who do not fall into neat categories or do fashionable things.

What effect will the new faces have?

from Colin Norman, Washington

THE results of last week's elections in the United States are simple to relate. The Democrats strengthened their hold on Congress, and they now occupy more than two-thirds of the seats in the House of Representatives and three-fifths of the seats in the Senate. (All members of the House of Representatives were up for re-election and one-third of the Senators.) The new Congress, which takes office in January, will be younger and probably more liberal than the present one, and there will be more new faces on Capitol Hill than at any time since the Second World War.

What do these changes mean for science? It is important to bear in mind two factors. The first is that because of the so-called seniority system, under which committee chairmanships are given to the longest serving members of the majority party, the newcomers will have little direct power. Few committee chairmen will be leaving this year and therefore the people who wielded the power in this Congress will continue to pull the strings in the next. The second factor is that party labels don't necessarily mean much when it comes to voting on specific issues since there is a wide spread of political opinion among members of both parties.

Nevertheless, there are a few changes in important positions on committees which deal with science and technology, with the Joint Committee on Atomic Energy being the most directly affected. Six out of the eighteen members of that committee either lost in last week's election or will be retiring at the end of this year, and the departures include two of the most influential members—Chet Holifield and Craig Hosmer. Since virtually every member of the joint committee is now a firm supporter of nuclear power, if even as few as one or two of the vacancies are filled by environmentalists or other nuclear sceptics the result would be important in changing the way the committee conducts its affairs.

Another significant departure is that of John Davis, the Chairman of the House Subcommittee on Science, Research and Development, who lost a primary election earlier this year. That subcommittee is the focal point for deliberations on general science policy matters in the House of Representatives and it also oversees the workings of the National Science Foundation. Davis's place will probably be filled by James Symington, a young and widely respected legislator from Missouri who

can be expected to step up the pace of the subcommittee's activities. Davis was also a member of the governing board of the Office of Technology Assessment and was in line to be its chairman.

Aside from direct dealings with science and technology, the new Congress is expected to be more sympathetic to environmental concerns because several Congressmen and Senators opposed by environmental groups were defeated. Among them were eight Congressmen listed earlier this year as the House's "Dirty Dozen" by a group called Environmental Action. In addition, twelve of seventeen candidates supported by the League of Conservation Voters were successful in the polls.

Those results have been greeted by environmental groups as proof that, in spite of the present concern about energy shortages, environmental issues are still politically potent. In particular, the results from Colorado indicate that environmental concerns there played a strong part in the election of a Senator, the Governor and at least one member of the House of Representatives. The upshot could be a setback to the government's plans to promote the extraction of oil from shale and the production of natural gas from deep deposits under the Rocky Mountains.

In the Senate election, Gary Hart, who was Mr McGovern's campaign manager in 1972, defeated Senator Peter Dominick after lambasting Dominick's voting record on environmental matters. As for the governorship, a state legislator, Dick Lamm, who had strongly supported laws to control urban growth in Colorado, defeated the incumbent governor. And in addition, Colorado voters approved a proposition on their ballot papers that before any more underground nuclear blasts can be set off in that state as part of a programme to extract natural gas from tight deposits, a referendum must first be held. The result of that move is likely to be the final blow for the Atomic Energy Commission's Plowshare Project.

Furthermore, environmentalists have hailed the election of other state governors, such as Edmund Brown in California and Edward Herschler in Wyoming, who are considered to be sympathetic to their views.

The election results may also portend some changes in the area of health, since many Congressmen supported by the American Medical Association (AMA) were defeated. One possible result may be to improve the chances that Congress will give its approval in the next two years to a meaningful national health insurance scheme. The AMA, which is vigorously opposed to

anything but a minimal health insurance scheme, poured about \$1 million into the campaign chests of Congressmen who supported its policies.

Perhaps the over-riding concern during the election campaign, however, was inflation and that will also have a direct bearing on the way in which Congress deals with some scientific matters. The Democrats have now been given a strong electoral mandate to curb inflation and they will be likely during the next two years to keep a strong hand on the federal government's purse strings, with the result that few controllable items in the budget will be allowed to grow. Energy research and development can be expected to escape the worst of the squeeze and so, perhaps, can such politically sensitive areas as cancer research. But for the rest of the science budget, a period of austerity should be anticipated. □

Soviet failure on the moon

from Vera Rich

ONE of the happier results of Soviet-United States cooperation in space research must surely be the increasing openness of the Soviet planners about their failures. In the early days, the possibility of failure did not exist—aborted interplanetary craft were discreetly registered under the cover-all of the Kosmos programme, and probes which failed to send back data were said to have "completed their mission" by the mere fact of effecting a landing or fly-by. The announcement of the Luna-23 failure comes with rather startling frankness.

True, 1974 has not been one of the most spectacular years for the Soviet space programme. Three out of four Mars probes failed to operate on reaching the planet, and the rather coy manoeuvring of the manned Soyuz-15 around the unmanned Salyut-3 in August caused wide speculation that a link-up had been originally intended. In these cases, however, the TASS reports did suggest certain positive achievement. However, Luna-23, launched on October 28 in good time for the Revolution Day celebrations, and hailed as part of the festal "illuminations" in a *Pravda* article of November 6, seems to have been officially recorded as a failure. Intended to carry out drilling operations to a depth of 2.5 m, it touched down in a rough area of the Mare Crisium, wrecked its drilling gear, and, after transmitting data for three days (which coincided neatly with the Revolution holidays) ceased functioning. □

correspondence

Mosquito research

SIR,—The article by Narender K. Sehgal (*Nature*, September 20) entitled "Doubts over US in India" is misleading and misinformed. The article repeats some of the allegations which have been made during the past few months in the Indian press about current mosquito research in Delhi, but it omits many subsequent retractions in the same press.

The Research Unit for Genetic Control of Mosquitos in Delhi is a joint venture of the Indian Council of Medical Research and the World Health Organisation. The unit was established about five years ago to study the ecology of three of the major disease vectors of India, *Culex pipiens fatigans*, *Aedes aegypti* and *Anopheles stephensi*, and to investigate genetic mechanisms which might be utilised for their control. All three are difficult to control by conventional methods, not only in India but throughout their distribution range. The research programmes were planned in India by ICMR and WHO scientists who, collectively, represented a range of disciplines and who shared a wide experience of vector-borne diseases. It is clear from the erroneous and misleading statements on filariasis, dengue and malaria that neither Mr Sehgal nor his informants are qualified to judge the scientific aspects of the programme, which makes it all the more reprehensible that they themselves have not taken the "closer look at the related details" of the "seemingly scientific investigations" which they recommend to others. The details are readily available. A whole issue of the *Journal of Communicable Diseases* published in the middle of 1974 in Delhi describes the programme. There is no secrecy, as has been charged, about the work. Having been closely associated with the unit and its staff for five years, both as a consultant and Project Leader, I can give this assurance unreservedly.

The main charge in the controversy, unfortunately still current, is that the data being collected on mosquito ecology may be, or are being, misapplied in some fashion relating to biological warfare. The same charge could equally well be laid in India and elsewhere against work being done on insect vectors of plague, viral encephalitis and typhus. The results from a great deal of biological and medical research could in theory be misapplied,

but this is a poor argument for criticising studies specifically designed for the long-term benefit of the community. If the interpretation of current mosquito research presented by Mr Sehgal is genuinely believed by some non-medical Indian authorities, then there is clearly a sad lack of understanding of the integrity and objectives both of local and foreign scientists in India and of international agencies.

There is nothing unusual about the financial support of the mosquito unit from US PL480 Indian funds. Scores of other research projects in India carried out by Indian scientists have been supported from the same source. The political implications that have been made have damaged relationships within the medical science community, and their continuance can only hinder progress in controlling mosquito-borne disease.

Sir Ronald Ross often expressed his frustration and discouragement when his research in India on *Culex pipiens fatigans* and bird malaria, which subsequently won him a Nobel prize, was misconstrued and retarded by the authorities of his day. It will be disheartening if, 75 years later, his successors suffer the same discouragement.

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Protests

SIR,—My letter published in your issue of August 12 set out the unequivocal policy of the World Federation of Scientific Workers on the rights of scientists to work and the procedure for implementing this policy in specific cases. Professor Janouch (*Nature*, September 20) does not approve of our policy and believes that "only open, public stands, protests and statements are meaningful and helpful". My own experience in working in international organisations over many years has taught me that, from the point of view of helping individuals in difficulty, not less than preserving the integrity of the organisation, an informal, low-key approach may often be more effective than the attitude he advocates.

Surely, also, before making protests and statements one should seek to discover whether the facts of the case have been fully and accurately presented. This is what our procedure tries to do and often, as in fact has hap-

pened in the particular case that led to this correspondence, new facts show the matter in a somewhat different perspective.

The case of Professor Holzer, referred to in Dr Janouch's letter, is much more serious than he claims because it is based on a law existing already in certain West German Länder and currently being prepared for submission to the Federal Parliament, whereby a whole category of people, members of certain legal parties and organisations, such as the German Communist Party (DKP), can be debarred from employment in public service posts, including posts as school teachers, judges and university teachers.

Dr Janouch also mentions the case of Academician Ivan Malek, who gave dedicated and distinguished service to the Federation over many years. Those who have heard or read addresses I have made to meetings of the Federation in recent years will be in no doubt about the high esteem and affection in which he is held in the Federation. Academician Malek was an active supporter of Alexander Dubcek and lost his posts as Director of the Institute of Microbiology in Prague and as a Vice-President of the Czechoslovak Academy of Sciences in the aftermath of the events of August 1968. Together with other officers of the Federation I made representations on his behalf at a high level with a view to ensuring that necessary facilities to enable him to continue his scientific work were made available to him. Although, however, he continued to work in the Institute he was not restored to his former position. According to information supplied to me from official quarters his work at the Institute terminated last autumn when he was three years past the usual retiring age of 60 for scientific workers who do not hold university chairs.

Academician Malek is a most distinguished and talented microbiologist who, in my opinion, still has very much to give both to Czechoslovakia and to world science. Speaking personally, therefore, I would deplore a regulation obliging a scientist to retire while still capable of first class scientific work no matter in which country it occurred. Nevertheless, one must surely concede that questions such as retirement age in a particular country are matters for the people of that country to determine.

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news and views

Towards chemical topology

THE uninitiated, faced with the awesome variety of organic chemistry, its peculiar, cryptonymous nomenclature and its tendency to produce publications in twenty or thirty parts, might be excused for finding there more to excite the wonder of the natural historian than the rigours of mathematics. Neither is the practitioner sketching formulae in the odorous intimacy of the laboratory likely to ascribe much abstract significance to the cyphers which he learns to manipulate as by second nature. Yet the business of classifying and labelling the complicated ring and branched structures which figure so prominently in the modern subject has been recognised in recent years to involve far more than simple book-keeping; in fact some of the problems which arise, when stripped to their abstract essentials, connect with as yet unresolved difficulties in the area of finite mathematics usually termed graph theory.

Consider, for example, the following: can a coding system be devised which not only leads to the systematic reconstruction of an arbitrary carbon skeleton but which is essentially independent of the particular way adopted for labelling individual atoms or other distinguishing features? Alternatively, if a certain labelling system is adopted (usually, of course, that of attaching sequential numbers to the carbon atoms present) can coded representations be found from which the topological identity of two differently numbered versions of the same N -carbon structure can immediately be inferred? (Other, that is, than by investigation of all possible $N!$ permutations of labelling.)

This problem, which shares its graph-theoretical content with several others in statistical mechanics and the theory of disordered structures, has been taken up in a number of recent papers, most notably by M. Randić of the University of Zagreb writing in the *Journal of Chemical Physics* (60, 3920; 1974). Professor Randić advances no rigorous proofs but suggests an ingenious algorithm by which a binary number code is assigned to each possible labelling of a given structure, the lowest number obtainable under systematic rearrangements providing both a canonical numbering scheme and an immediate comparison test for the identity of different networks of bonds. The method given seems to fall short of guaranteeing the discovery of the minimal labelling but seems to work well when tried on the common systems of saturated and benzenoid hydrocarbons. Needless to say the canonical numbering systems come out differently from those traditionally adopted. (It is notable, and amusing, that the basic tool of the Randić method—the adjacency matrix for comparable structures—is now a familiar part of O-level mathematics (see *Schools Mathematics Project Book Y*, 55; Cambridge University Press, 1973) though likely to seem formidable to many research chemists educated in an earlier era.)

The recent involvement of stereochemistry with topics such as graph theory, the less usual point-group geometries, the interconversion of symmetry types and the combinatorics of multiple structures, is an encouraging sign that this subject, cutting across all the main disciplines of chemistry, might be prominent in any defence of the latter

against charges of moribundity and compartmentalism.

A very timely cross section of contemporary (organic) stereochemical research, as well as a useful reminder of the heroic era of the subject is to be found in the recent van't Hoff–Le Bel centennial issues of *Tetrahedron* (30, Nos 12, 13; 1974). Here some fifty papers are collected covering topics so various, and occasionally bizarre, as to make selective quotation at best misleading. Among the less orthodox papers are those on "Polytopal Form and Isomerism" (E. L. Muttart, Cornell), "High Symmetry Chiral Molecules" (M. Farina and C. Morandi, Milan/La Jolla), "Polyhedral Rearrangements Involving 5 and 6-coordinate Carbon" (M. F. Hawthorne, K. P. Callahan and R. J. Wiersma, UCLA), "Knot-structures in Chemistry" (J. Boekmann and G. Schill, Freiburg), "Parity and Stereochemistry" (J. Mathieu and A. Rassat, Grenoble) and "Chemical Systematics in the Computer Design of Syntheses" (J. Blair, J. Gasteiger, C. Gillespie, P. D. Gillespie and I. Ugi, Munich). The last mentioned also involves the graph theoretics of labelling, the time in the enumeration of synthetic pathways by computer. Many exotic systems make an appearance—cyclophanes, propellanes, carboranes—among others less strange though scarcely worked out stereochemically, such as tetra-aryl methanes. A disappointment perhaps is the non-appearance of the marvellous and unique dodecahedrane, which several groups have been rumoured to be close to synthesising, perhaps the most beautiful hydrocarbon that will ever be made.

Yet the outsider looking in at such intricate and painstaking work cannot escape certain mixed feelings. Granted that there is a place for *jeux d'esprit* in such a collection, how important is it to the future of chemistry that someone should succeed in synthesising a molecule with a knot or a peculiar kind of hole in it or a particularly awkward linkage of rings? And, if dodecahedrane can be made, should we take comfort in the fact that rhombic dodecahedrane is manifestly impossible? In short, are these weird and wonderful aspects of stereochemistry outgrowths of a flourishing field, or hot-house creations of almost zero relevance to either the theory or the application of the subject? Should one not, after all, admit that chemistry has become little more than a complicated and private 'bead-game'? The riposte to this no doubt lies in pointing to the considerable achievements of mainstream stereochemistry—conformational analysis, the Woodward–Hoffmann rules, and the various aspects of 'kinetic stereochemistry'—which have come into their own recently, while stressing the underlying biochemical relevance of much (though by no stretch of the imagination all) that has been done. This line is taken in a recent interview given by Alexander Todd (*Chemistry in Britain*, 10, No. 6, 207; 1974) who argues with impressive conviction, if not altogether convincingly, that with sufficient imagination and resources, genuinely new and relevant phenomena will continue to be found. Given that it is not an entirely happy thing that chemistry should be in a position of needing a 'something will always turn up' philosophy, only time and a great deal of work will prove—or disprove—such a contention.

Meanwhile, one might argue tentatively that the move towards interest in global structure, neighbour relationships and the problems of chemical identity touched on above are a proper, if delayed, response to the realisation that the

truly basic problems of stereochemistry at the geometrical level were solved a very long time ago indeed by van't Hoff, Le Bel, Kekulé *et al.* and that a great deal of what has followed is elaboration rather than fundamental advance. Perhaps we shall know that something extraordinary has arrived when the authors of a radically new concept receive the kind of abuse which was heaped upon van't Hoff and Le Bel by chemists of the day, for daring to suggest that carbon atoms might combine in geometrically describable structures. But, if one's instincts are correct, this honour may well be reserved for someone in a field altogether remote from chemistry as we know it, and perhaps from physics too.

from our Molecular Physics Correspondent

New light on old reflexes

THE voluntary muscles that move our limbs are full of sense organs, a fact in itself not surprising, although the use the central nervous system makes of the information they send it is perhaps more of a mystery than is the case for any other major group of sense organs in the body.

The most obvious manifestation of their action, the familiar knee jerk, obtained by striking the tendon below the knee cap with a hammer, was described independently and almost simultaneously by Erb of Heidelberg and Westphal of Halle just on a century ago. In this reaction the tap to the tendon briefly stretches the muscle spindles (the most important of the sense organs in the muscle) and their discharge reflexly causes the muscle to twitch. Slower stretch of a human muscle that is already contracting, by forcible bending of the joint it acts on, also causes a reflex increase in contraction. Stretch reflexes of this general type were first described by Liddell and Sherrington in 1924 in animals rendered insentient by removing the cerebral hemispheres, whose muscles are unnaturally susceptible to stretch. The stretch reflex has long been a favourite object of research from the belief that it is an important and accessible element in the complex nervous mechanisms by which the muscle sense organs make the muscles, by some kind of feedback action, do what we ask of them. This belief is reinforced by the observation that stretch reflexes, as well as tendon jerks, are abnormal in neurological diseases such as stroke, in which the muscles conspicuously do not do what we ask of them.

The muscle spindles are the most elaborate sensory structures in the body other than the eyes and ears. Each consists of a bundle of fine modified muscle fibres with sense endings, sensitive to extension of these muscle fibres, wrapped around a central region. There are two different kinds of sense endings on the spindles, the primaries, connected to the central nervous system by fast conducting nerve fibres, and the secondaries, whose nerve fibres conduct at only a half or a third of the speed.

The latency of the knee jerk—the time from contact of the hammer with the tendon to the first sign of reflex activity in the muscle extending the knee—is about a sixtieth of a second, so short that the sense endings responsible must be the primaries with their fast nerve fibres. Even so, there is only just time for nerve impulses to get to the spinal cord and back. Until quite recently it was always supposed, following Sherrington's unhesitating identification of the knee jerk as a "fractional manifestation" of the stretch reflex, that the stretch reflex proper used the same sense endings and the same rapid spinal reflex pathway as the tendon jerk. This attractive but dangerous simplification is now under attack on two fronts.

First, P. B. C. Matthews discovered that the stretch reflex in the soleus (a muscle extending the ankle) of the cat was larger than the reflex contraction obtained by vibrating the tendon, a mode of stimulation that is believed on good grounds to excite the spindle primary endings powerfully

and selectively. This strongly, if indirectly, suggests that the secondaries take part in the stretch reflex. There is no reason why they should not, for the stretch reflex has long latency components as compared with the tendon jerk. Now Kirkwood and Sears in this issue of *Nature* (page 242) present the crucial evidence that the nerve fibres from spindle secondaries, on entering the spinal cord, excite directly (monosynaptically) the motor nerve cells of the muscle in question (in these experiments a muscle of the rib cage). They have similar evidence for the secondary endings in cat soleus muscle (*J. Physiol., Lond.*, in the press). The involvement of the slow-conducting spindle secondaries in the stretch reflex of the decerebrate cat can now scarcely be doubted.

On the second front, Marsden, Merton and Morton (*Nature*, 238, 140; 1972; *Lancet*, i, 759; 1973) working on human subjects, found that in some muscles, for example the muscle that bends the top joint of the thumb, only long latency components are present in the stretch reflex. Following suggestions by Hammond and by Phillips, they argued that in such muscles the stretch reflex may not be spinal at all, but may travel over a pathway to the cerebral cortex and back. This theory may seem far-fetched at first sight, but trans-cortical reflexes from the limbs are not, in fact, a new idea in either physiology or neurology. A number of observations already fall into place on the trans-cortical theory of the stretch reflex, but much will have to be done to establish the theory firmly. In particular, there remains at present the possibility that the latency is long because only the spindle secondaries (to the exclusion of the primaries) take part in a spinal reflex. Results from muscles of the shoulder should throw light on this question.

These two new notions, that the secondary spindle endings take part in stretch reflexes and that some stretch reflexes may have a cortical reflex arc (notions, incidentally, by no means mutually incompatible), have a twofold significance. First, in clinical medicine, neurologists have long been puzzled that the stretch reflexes (measured as the resistance or 'tone' felt in a limb during passive movement) and the tendon jerks, which physiologists originally told them had the same mechanism, did not always go hand in hand when they altered in disease, and sometimes indeed changed in opposite directions. Now that there are at least two possible ways in which the mechanism of tendon jerks and stretch reflexes may differ, the way is open for a resolution of these problems and for a completely new approach to the whole nature of 'tone' in normal muscle and its alterations in spastic and other abnormal states.

More generally, any new information about the detailed mechanism of the stretch reflex is particularly welcome at this time when the work of Marsden *et al.* already referred to on the servo-like stretch-reflex-based responses of human muscles during voluntary movements has turned up several other new phenomena not foreshadowed in animal work on the stretch reflex and badly in need of explanation.

from a Correspondent

An uphill route for climatic cycles?

If climate is the summation of weather over a period of time, then it makes sense to predict climatic changes in the same way as weather—that is by looking at present sequences of events and assuming that similar sequences will persist in the future. Such a method has been applied principally by Lamb and it is one of the concerns of his now rescued Climatic Research Unit (*Nature*, 251, 568; 1974). But the detection of cycles alone does not indicate the causes of either regional or global climatic changes. As realistic experiments are clearly impossible and

computer models cannot yet account for present climatic features, never mind their changes, the only practical approach is to define as closely as possible the nature and wavelength of climatic cycles and to compare them with any cyclical phenomenon that may alter climate. Lamb, for example, demonstrated that there was no strong correlation between climatic change and volcanic activity (*Phil. Trans. R. Soc.*, **A266**, 425; 1970). If correlations can be established, causal relationships can be investigated bearing in mind that the two factors could be indirectly linked through some other variable.

But climatic records are of only limited range and difficult to quantify—Tacitus's description of the British climate in 75 bc as "objectionable, with frequent rains and mists, but no extremes of temperature" bears distinct resemblances to the British summer of 1974 AD, but cannot be taken to mean that we are at the same point of a climatic cycle! The only quantitative climatic measure for periods prior to meteorological measurements is that of temperature determined from the ratio of ^{18}O to ^{16}O now trapped in ice on Greenland and Antarctica and in skeletal remains preserved in sediments. This ratio can be measured with a high precision, but both situations raise difficulties about how the ratio should be interpreted as temperature. The snowfall over the Antarctic comprises a large amount of redeposited snow and the oxygen ratio in marine skeletal organisms is strongly affected by changes in salinity as well as by temperature. The oxygen ratios in ice sheets can be dated by short-lived isotopes or simply counting summer/winter layers—usually starting from the

commencement of nuclear weapon testing which now provides an easily detectable marker horizon. But increasing compaction with recrystallisation tends to destroy the accuracy of counting and this method can only go back reliably for a few thousand years. Fossil foraminifera and other organisms can however be used to extend temperature determinations well into geological time and can be dated by standard geological methods, thereby allowing the detection of long-term cycles.

What about the factors that may alter climate? As Chappell says on page 199, there has been general acceptance that cyclical changes in the degree of solar radiation received at the Earth's surface occur because of long-term changes in the Earth's orbit. The periodicity of these changes was largely established by Milankovitch in 1938 and, assuming no changes in the solar radiation 'constant', it is possible to determine precisely the changes in the amount of insolation received at the Earth's surface. Calder, on page 216, re-evaluates Milankovitch's hypothesis in the light of revised insolation tables and concludes simply that these orbital perturbations are the first-order control on glaciation. But as Chappell points out, many critics have claimed that the radiation changes associated with these astronomical factors are very small and the direct effect of insolation may be insignificant compared with indirect factors. Chappell has previously shown many earlier correlations to be spurious, but now provides new and revised evidence for a degree of correlation between orbital perturbations, sea-level changes and palaeotemperatures for the last 250,000 years. His conclusion is also that

More putative human tumour viruses

In this issue of *Nature* (page 247), McGrath, Grant, Soule, Glancy and Rich describe the release from a human breast carcinoma cell line (MCF-7) of virus particles that they believe may be human mammary tumour viruses. The particles have all the standard biophysical and biochemical properties of RNA tumour viruses, and virus-producing cells react specifically in indirect immunofluorescence tests with antiserum against murine mammary tumour virus. These findings support previous reports by Schlom, Axel, Spiegelman and their colleagues and by Moore's laboratory that virus particles related to murine mammary tumour viruses can be detected in human milk and in breast carcinomas. Unfortunately virus production from the MCF-7 cell line is rather poor and fickle, and this has hampered a more thorough analysis of the particles.

The sceptical reader will wonder whether the MCF-7 cells are really human cells and if so whether they are really derived from a breast carcinoma rather than a common laboratory cell, such as HeLa. On these points, McGrath *et al.* stand on reasonably firm ground, for in a previous publication (*J. natn. Cancer Inst.*, **51**, 1409; 1973) Soule, Fazquez, Long, Albert and Brennan characterised the MCF-7 cell line in some detail. MCF-7 cells are derived from cultures of a pleural effusion of a patient with metastatic mammary carcinoma. The sub-tetraploid cells seem to have a human chromosome pattern, although banding patterns have not been examined; they have a ribosomal RNA profile resembling that of human cells, and they carry human cell surface antigens and human glucose-6-phosphatase isozyme. MCF-7 cells show three features characteristic of mammary gland cells: cytoplasmic receptors for 17β -oestradiol, synthesis of lactalbumin, and the formation in monolayer culture of epithelial 'domes' similar in morphology to those previously described by McGrath in murine mammary tumour cultures. Not all subclones of the MCF-7 cell line release virus particles. There

remains the possibility, of course, that although the cells seem to be genuine human mammary cancer cells, the virus may be a laboratory contaminant. But McGrath *et al.* feel that they can distinguish the MCF-7 particles from murine mammary tumour viruses, and there is no relationship at all with murine leukaemia virus.

In a paper published in the November 1 issue of *Nature* (252, 78), Lewis, Tannenbergh, Smith and Schwartz suggest that an antigen detectable on lymphocyte membranes of systemic lupus erythematosus (SLE) patients is related to a C-type viral antigen. Antiserum prepared against the C-type virus produced by murine plasmacytoma SP 104 reacted in immunofluorescence tests to SLE lymphocyte membranes, but not to lymphocytes of normal subjects. Moreover, the serum of an SLE patient also reacted to SLE lymphocytes and this reactivity was specifically removed by absorption with gradient-purified SP 104 virus. Thus there seems to be a common antigen between SP 104 virus and human SLE cells.

SP 104 cells have a curious history and interesting properties. The plasmacytoma arose following inoculation of cell-free filtrates of canine SLE cells into CAF₁ mice (Lewis *et al.*, *J. clin. Invest.*, **52**, 1893; 1973). Yet the SP 104 virus stock includes a leukaemia virus containing murine gs-1 antigen. The SP 104 cells produce antibody against double-stranded DNA, as is found in SLE patients, and inoculation of SP 104 virus into normal mice or dogs induces the appearance of anti-nuclear antibodies. Thus the canine cell-free extracts and the SP 104 virus induce symptoms resembling SLE in recipient hosts. It is not known whether SP 104 virus contains canine as well as murine components; neither is it known whether the relation of SLE cell membrane antigens to viral antigens is specific to SP 104 virus alone. Nevertheless these studies do implicate a virus in the aetiology of SLE, and they suggest that the human, canine and experimental murine forms are closely related.

R. A. WEISS

astronomical factors are of prime importance, but the way in which world-wide climatic changes occur are very much more complex than indicated by Calder.

Such considerations are not merely of academic interest—they become vital as large-scale technology begins to alter the environment. What is the likely climatic effect of diverting Soviet Arctic rivers southwards for irrigation projects? What would be the effect of a permanent ice-free channel for the movement of oil from Arctic Canada? What is the climatic effect of pollutants in the stratosphere? These questions will not be answered until a better dating system for the Quaternary enables the various cycles to be defined. Then the statistical validity of the many possible correlations can be tested and their causative relationship evaluated.

D. H. TARLING

Plants with talent for breeding or competing

from Peter D. Moore

POPULATION ecologists have made considerable use of the concept of r and K selection, which was introduced by MacArthur and Wilson (*The Theory of Island Biogeography*, Princeton University Press; 1967). When intraspecific competition (and, therefore, density-dependent mortality) is low, natural selection operates chiefly through its density-independent component, r selection. Taxa associated with such situations are said to be r selected. Conversely, when intraspecific competition and density-dependent mortality are high, K selection is operative. Theoretically, r selection should favour organisms which can breed rapidly, and K selection those which can most efficiently exploit a resource. Pianka (*Am. Nat.*, **104**, 592; 1970 and **106**, 581; 1972) has pointed out that no organism is completely r selected or K selected, but lies at some point along a continuum between these extremes.

Investigations into selective pressures in populations of organisms have mainly concentrated upon animals, but Gadgil and Solbrig (*Am. Nat.*, **106**, 14; 1972) suggested that selective pressures took a similar form in populations of dandelions (*Taraxacum officinale*) in different types of grassland at Ann Arbor, Michigan. Since this species is extremely plastic in its morphology it was necessary to use isozyme patterns in leaf extracts to determine genetic biotypes. Four such variants were found, one (A) being associated with the most highly disturbed situations, another (D) being found in stable, undisturbed situations and the other two being intermediate. The series seemed to show a trend from r to K selection.

Solbrig and Simpson (*J. Ecol.*, **62**, 473; 1974) have now published some experimental data which support this suggestion. Taxa which have been subjected to r selection would be expected to expend a larger proportion of their energy resources in reproductive effort in order to exploit rapidly an unsaturated (low competition) environment. Energy expended per seed, however, need not be high because of the lack of intraspecific competition. Biotype A was found to produce an average of 25.3 seeds per plant with a mean seed weight of 0.32 mg; biotype D bore 8.2 seeds per plant with a mean seed weight of 0.44 mg. These extreme biotypes thus fulfil the theoretical requirements of r and K strategists in this respect. One would further expect a K -selected taxon to be a more effective competitor under high density conditions than an r -selected one. This was tested by growing the two biotypes both alone and in combination. When in pure culture there was no significant difference between the biotypes in terms of dry weight increase over 90 weeks. When grown together, however, type D always produced the heavier plants and had the lower mortality rate; it can therefore be regarded as the stronger competitor of the two.

These two extreme biotypes thus demonstrate adaptation apparently in response to differing pressures of r and K selection in their two sites. It is not possible, however, on the basis of these experiments, to determine whether these forms of selection are the most influential ones at work upon the dandelion genomes. Habitats in which r selection is operative are frequently subjected to severe edaphic and microclimatic stresses which could have an overriding selective influence on survival. K -selection situations on the other hand experience high levels of interspecific as well as intraspecific competition, which could call for rather different adaptive mechanisms. The apomictic nature and therefore the genetic homogeneity of dandelion biotypes will make them ideal material for the investigation of these further problems.

Meteor rates, volcanoes and the solar cycle

from David W. Hughes

A WORLD-WIDE increase in radar meteor echo rates occurred in 1963 and the cause of this has been puzzling scientists ever since. These echoes are obtained by reflecting short (100 μ s) pulses of radiation (in the tens of MHz region) from the ionised trains produced in the

upper atmosphere (at heights of 80 to 100 km) by incoming meteoroid particles. The number of echoes seen in 1963 was a factor of 1.5 to 2 greater than in previous and subsequent years and this increase was recorded in Christchurch, New Zealand¹, Ottawa, Canada² and Onsala, Sweden³, so instrumental effects can be ruled out. The effect was periodic, in New Zealand occurring during the winter (June, July) and recurring on successive years with reduced magnitude. In the Northern Hemisphere the effect occurred initially in May 1963 but on following years occurred in the winter months, January–March. The Swedish data showed a rate increase for all echo duration. Canadian data however indicated that the anomalous increase was restricted to the smaller meteors. The rates were also proportionally higher throughout the day⁴.

Kennewell and Ellyett of Newcastle University, New South Wales have reconsidered the possible causes of the rate increase in a recent article in *Science* (**186**, 355; 1974). They divide the possible causative agents into extraterrestrial and terrestrial.

Consider the extraterrestrial possibility first. There are no reasons why the influx of meteoroid dust to the Earth should not vary from year to year. For example certain meteor showers are distinctly periodic in their activity, the Leonids and Giacobinids being perfect examples. Showers are fed by the decay of the parent comet and it takes a considerable time for the new meteoroids to move around the comet orbit and form a complete, uniformly dense toroid of dust in space. The loop formation times of the Quadrantid and Perseid meteor streams are for example 330 and 4,000 years respectively. The activity of streams which are young and not completely formed is expected to vary with the periodicity of the original comet (33 and 6 years respectively for the Leonids and Giacobinids) the dust still being closely grouped around the comet. A considerable proportion of the sporadic meteoroid background (~30%) is made up of minor streams and therefore the mean periodicity of these streams might be reflected in the total influx rate. But the 1963 increase extended over a period of some months which is incompatible with the period of activity of most meteor showers, usually less than a month. Also the fact that the recurring annual variations after 1963 were 6 months out of phase in opposite hemispheres cannot be explained. And the form of the monthly mean diurnal variation remained unchanged—this being something which would vary decidedly if a shower was active. Kennewell and Ellyett conclude that the rate increase in 1963 was not

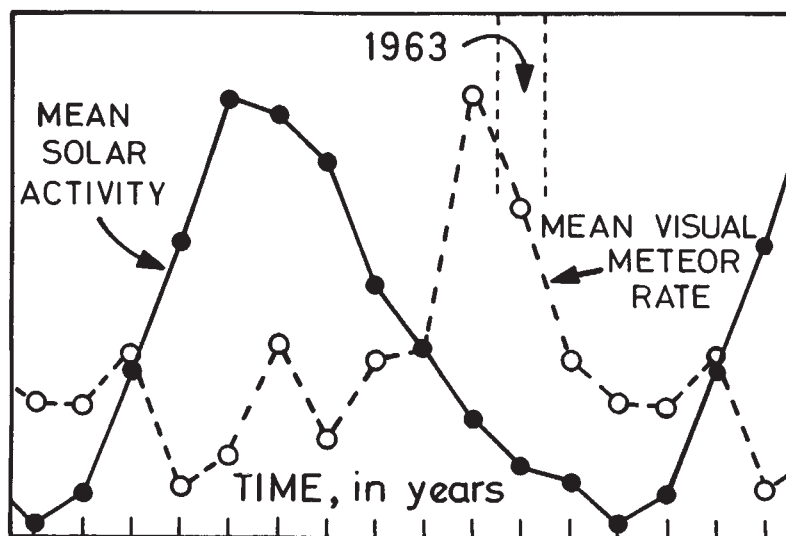


Fig. 1 The mean visual meteor rate as a function of time throughout a solar cycle. The rate increases by a factor of two for the two years near solar minimum, being approximately constant over the rest of the cycle.

caused by an increased particle influx.

Is the cause terrestrial? Did the properties of the upper atmosphere change in 1963, resulting in the improved detection of meteors? The two main atmospheric parameters which affect meteoroid ablation are density and scale height. Now a uniform increase in atmospheric density down to meteor heights would cause no increase in the number of meteor echoes since the entire meteor population would simply rise in height. This would change the average range of the echoes. McIntosh⁴ found a change in range of about 3 km in 160 km in 1963, a change which would have an insignificant effect on the rate. The initial radius of a meteor train varies as a function of height, also the reflected power from a train decreases exponentially with its radius. This produces a height ceiling effect, the echo height distribution being curtailed at the top. Smaller meteoroids which burn out at these great heights will be observed in greatly reduced numbers. But a simple increase in density just raises the height ceiling with the mean height, while the observed rate remains the same.

If the density gradient varies, as a result, for example, of a change in scale height the effect is more complex. First a larger train electron line density will result from a particular meteoroid thus allowing smaller meteoroids to come within the radar detection range. Second, a decrease in scale height will decrease the length of the train, making it more difficult to detect and will tend to cancel out the previous gain.

Kennewell and Ellyett propose that the meteor rate increased because the scale height around 90 km changed after the massive injection of volcanic dust into the upper atmosphere by the eruption of Mount Agung on the island of Bali (8°S, 155°E) in March 1963.

This dust was mainly concentrated at a height of 20 km but also formed a minor layer in the mesosphere where, by absorbing solar radiation, it led to the heating of the immediate atmosphere, a lowering of density in the region and a subsequent increase in the atmospheric density gradient higher up. The lag between the southern and northern hemisphere rate increases is thought simply to be due to dust transport times. Kennewell and Ellyett also show that total atmospheric dust content measurements made in Australia correlate with the meteor rate and that the biennial oscillation of the equatorial stratospheric winds could inject small particles into the mesosphere on alternate years and produce a two-yearly variation in meteor rates.

A cautionary note must however be sounded because 1963 is not the only year of excessive meteor rates. As Ethiopian rainfall, thunderstorm activity and the quality of claret vary with the solar cycle why shouldn't the meteor rate? This question was answered in the affirmative by the Czechoslovak astronomer Bumba in 1948 (*Bull. Astr. Inst. Czech.*, **1**, 93; 1949). He calculated, among other things, the mean annual rate of visual meteors during the period 1844–1933 as a function of position in the solar cycle and his results are shown in the figure. He also found that the ratio of bright to faint meteors and the mean observed geocentric velocity⁵ varies with the solar 11-year periodicity. There is an inverse correlation between solar activity and visual meteor rate, the rate maximising near solar minimum. This was also mentioned by Lindblad³ who found that the height of first appearances of meteors from a given shower remained around 110 km whereas the average end point rose from 85.2 km in 1956 to 96.0 km in 1963—a change of 11 km. Lindblad also

found the 1953 rate considerably higher than the average.

If the increase around 1963 is just a solar cycle effect (a deduction supported by the work of Bumba and Lindblad) and not a one-off affair caused by a volcanic explosion (and it can be seen from the figure that 1963 coincides exactly with visual rate increases seen between 1844 and 1933) then similar increases should be found at other times of solar minimum, for example during 1972–73. So now is an excellent time for visual observers to peruse their records to see if this increase occurred. Also the experts in meteor ablation theory and upper atmosphere physics should try and work out why the solar cycle-induced variation in atmospheric scale height has such an effect on observed meteor rates. They could also investigate whether some unknown factor affecting meteor physics and ion chemistry varies with an 11-year periodicity. As the meteor rates are proportionally higher during the day perhaps the decrease in solar X-ray and ultraviolet flux at solar minimum have an effect.

¹ Ellyett, C. D., and Keay, C. S. L., *Mon. Not. R. Astr. Soc.*, **125**, 325 (1963).

² McIntosh, B. A., and Millman, P. M., *Science*, **146**, 1457 (1964).

³ Lindblad, B. A., in *Physics and Dynamics of Meteors* (edit. by Kresak, L., and Millman, P. M.), 50 (Reidel, Dordrecht, 1968).

⁴ McIntosh, B. A., *Smithsonian Contr. Astr.*, **11**, 201 (1967).

⁵ Bumba, V., *Bull. astr. Inst. Czech.*, **6**, 141 (1955).

Pinned superfluid vortex

from P. V. E. McClintock

A QUANTISED vortex line stretched between definite pinning sites in superfluid helium has apparently been observed for the first time by Jost, Bogatin and Weaver of the Massachusetts Institute of Technology (*Physics Letters*, **49A**, 147; 1974).

Liquid helium four below its so-called lambda transition temperature at 2 K behaves in many ways as though it were a mixture of two distinct fluids: a normal component which has similar properties to ordinary liquids; and a superfluid component which, having zero viscosity, is able to flow effortlessly through channels of vanishingly small dimensions and is thus responsible for the well known and very peculiar properties exhibited by the liquid.

One of the oddest features of the superfluid component is that it cannot rotate in a conventional manner. There is excellent theoretical and experimental evidence showing that if a container of superfluid helium is slowly rotated, the liquid remains at rest with respect, presumably, to the fixed stars, no matter how long the experiment is

continued. If the angular velocity of the container is further increased, however, then it may become energetically favourable for one or more vortex lines to appear in the liquid. Each of these consists of a narrow core, oriented parallel to the container's axis of rotation, and around which the helium flows with a tangential velocity which is inversely proportional to the distance r from the core. The strength, known as the circulation, of each vortex is quantised in units of h/m where h is Planck's constant and m the mass of a helium atom but, so far, only singly quantised vortices have ever been detected.

Arguments based on minimising the total energy suggest that a single vortex line in a smooth container will lie along the axis of rotation, that two vortices will circle around each other under influence of their mutual flow fields, and that larger numbers of vortices will form a hexagonal lattice rotating with the container. But the recent elegant photographic experiments of Packard and Sanders (*Phys. Rev. Lett.*, **33**, 280; 1974) have shown that this does not seem to be the case in practice: the vortex cores seemed to be arranged in no definite pattern and indeed to wander aimlessly relative to the container while still presumably remaining parallel to its rotational axis.

The MIT group employed a container with a pair of definite pinning centres, designed to detect the appearance of a single vortex. The helium was contained in a 2.5 mm diameter cylinder at each end of which, positioned on the axis, was a needle, the two points being separated by about 5 mm. There were two reasons why it was expected that the first vortex would form between the needle points: first, even in a flat-ended container, a single vortex minimises its energy by being on the axis of rotation; and, second, by spanning the space between the needles, the vortex could be shorter, and thus of lower energy, than in any other feasible position. To detect the formation of a vortex line, one of the needles

was used as a field emitter, injecting electrons into the liquid, while the other acted as a collecting electrode. Because vortex lines are able to trap and hold electrons, it was hoped that there would be a sudden change in the collector current when the vortex appeared.

As the rotational speed of the chamber was gradually increased from zero, a discontinuous decrease in the collector current was indeed observed, and it occurred at approximately the expected angular velocity. Although the mechanism responsible for the fall in collector current is still not understood in detail, the authors ascribe the phenomenon to the formation of a singly quantised vortex line strung between the two needles. If they are right, then it will be the first time that a pinned vortex, long a familiar concept in connection with superconductors, has been observed in superfluid helium.

A particularly interesting suggestion by the MIT group is the possibility of using the same apparatus to search for a vortex line having two or more quanta of circulation. They argue that, if the separation of the needles is considerably less than the length of the chamber then, as the rotational speed is increased, it must be energetically preferable to add more quanta of circulation to the existing pinned vortex rather than creating further singly quantised lines which would have to span the whole length of the cylinder. Their preliminary attempts to detect such a double-strength vortex have been unsuccessful. This is perhaps because of the randomly orientated tangles of vortex lines which are believed to be generated by electrons in the large electric field close to a field emitter and which would be likely to interfere with the stability of the main, rotationally created, vortex. If this effect can be suppressed, however, perhaps by conducting the experiment under pressure when it is known that electrons have much less tendency to create vortex lines, some interesting new phenomena may be anticipated.

On the trail of epidemic hepatitis

from Arie J. Zuckerman

INFECTIOUS or epidemic hepatitis, now referred to as hepatitis type A, remains a major public health problem, occurring endemically in all parts of the world with frequent reports of small and large epidemics. Spread is usually by person to person contact and major outbreaks result most frequently from the faecal contamination of water and food. Subclinical cases are common; the disease has in general a low mortality but patients may at times be incapacitated for many weeks or months. Further general information on transmission experiments to human volunteers and studies in non-human primates may be found in a recent WHO report (*Viral Hepatitis: Report of a WHO Scientific Group; Techn. Rep. Ser.*, **512**; 1973).

The discovery of the association between an antigen found in blood and hepatitis type B (serum hepatitis) has prompted efforts to find a similar antigenic marker in patients with hepatitis type A. To date, a specific serum antigen has not been found but examination by immune electron microscopy of faecal extracts prepared from patients with acute hepatitis A yielded interesting results. An antigen was detected by immunodiffusion in faecal extracts from about 50% of patients admitted to hospital in Australia with hepatitis A and in only 2% of control patients (Ferris *et al.*, *Lancet*, **ii**, 243; 1970). Antigenic activity was associated with two types of particles measuring 15–25 nm and 40–45 nm in diameter. Animal antisera to this faecal antigen yielded encouraging results but also a number of non-specific reactions, and studies are currently in progress with antisera with improved specificity.

In December 1973, virus-like particles measuring 27 nm in diameter were demonstrated by immune electron microscopy in faecal extracts obtained from 2 out of 4 adult volunteers who were infected by injection or by



Painted panels from the ceiling of the Natural History Museum, London. According to *The British Architect and Northern Engineer* of June 1878 "the chief idea . . . is that of growth". Left to right: Scots pine *Pinus sylvestris*; peach *Amygdalus persica*; cacao *Theobroma cacao*; English oak *Quercus robur*.

mouth with the MS-1 strain of hepatitis A virus (Feinstone and colleagues, *Science*, **182**, 1026; 1973). The particles were heavily coated with antibody and they were aggregated by convalescent serum. These particles were not found in faecal specimens before infection, they were only detected at the onset of clinical symptoms, and pre-illness sera did not contain antibodies to the virus-like particles. Faecal filtrates containing these 27 nm virus-like structures were used to examine by electron microscopy several groups of sera for the presence of antibody. All 6 volunteers previously infected with hepatitis A developed serological evidence of infection as judged by aggregation and antibody coating of the 27 nm particles. Similar results were obtained with sera from patients with a number of naturally occurring outbreaks of hepatitis A. Other studies have shown that the buoyant density of the faecal particles in caesium chloride was 1.41 g ml^{-1} and together with the morphological appearance and known resistance to ether, acid and heat the data suggest that this particle is a parvovirus (Feinstone and associates, *J. Virol.*, **13**, 1412; 1974). Morphologically and antigenically similar particles were also demonstrated in faeces from patients from at least one geographically separated outbreak of hepatitis A (J. Maynard and colleagues, personal communication). The particles identified in Arizona banded, however, in two peaks in caesium chloride at 1.29–1.33 and at 1.39 g ml^{-1} . Preliminary data from experimental transmission to marmosets and susceptible chimpanzees have also demonstrated a close association between the faecal particles and hepatitis A.

On the other hand, it is generally recognised that faeces contain a multitude of bacterial, bacteriophage, viral and other particulate antigens. Almeida and her colleagues (*Lancet*, **ii**, 748; 1974) examined by immune electron microscopy and by immunodiffusion faecal extracts and acute and convalescent sera from an outbreak of hepatitis A. Faecal extracts reacted with convalescent sera contained frequent distinctive aggregates having the appearance associated with immune complexes. But particles resembling bacteriophage, particles resembling togavirus and a range of spherical particles were all present in distinct immune complex form. Serological analysis by gel diffusion showed seroconversion to more than one antigen present in the faeces, and seroconversion was also detected to antigens present in a number of control faecal extracts. It was also pointed out that it is known that antibody titres to bacterial, viral and dietary antigens are increased in patients with acute and chronic liver

disease and this may account for the multiplicity of immune reactions in viral hepatitis.

The latest results reported by Almeida and colleagues (*Lancet*, **ii**, 1083; 1974) after the examination of faecal extracts from one of the infected adult volunteers tested by Feinstone and associates (*loc. cit.*) confirmed the presence of the 27 nm particles, but at least two small cubic virus-like particles were found in addition. Using the technique of immune electron microscopy in London, extracts of faeces obtained 33 days after infection with the MS-1 strain of hepatitis A were found to contain virus-like structures which varied in their size. Both 'full' and 'empty' particles measuring 22 nm, 27 nm and 30 nm in diameter could be distinguished and they seemed to have a cubic symmetry. Each of these virus-like structures was clearly coated with antibody and the particles were aggregated by convalescent but not by acute serum from hepatitis type A. The fact that this material has also induced hepatitis in 2 out of 6 marmosets suggests that hepatitis A virus is indeed present. But the precise relationship to the disease of the three virus-like particles now described remains uncertain.

The success of immune electron microscopy in elucidating the complex antigenic reactivities of hepatitis B virus may blaze a trail for what seems to be an equally puzzling array of particles present in the faeces, and perhaps the serum, of patients with hepatitis A infection.

Eclogite model of upper mantle

from Peter J. Smith

THE view that the Earth's upper mantle might be eclogitic in composition was put forward over 60 years ago by Fermor (*Rec. Geol. Surv. India*, **43**, 41; 1913), although since then it has received comparatively little support. According to Green and Ringwood (*Phys. Earth Planet. Interiors*, **3**, 359; 1970), for example, "the weight of geological and geophysical evidence on upper mantle composition favours an overall peridotitic composition", and the arguments against an eclogitic composition were given by Ringwood and Green (*Tectonophysics*, **3**, 383; 1966). During the past decade, Green and Ringwood (see, for example, *Earth planet. Sci. Lett.*, **3**, 151; 1967) have given particular attention instead to their "pyrolite" composition, although the validity of this system is by no means universally accepted.

Interest in the eclogite model began

to revive in 1970 when Press (*Phys. Earth Planet. Interiors*, **3**, 3; 1970) used a Monte Carlo procedure to derive a series of Earth models consistent with geophysical data. Specifically, he found that of about 50 acceptable density models, about 30 required densities of at least 3.6 g cm^{-3} somewhere in the upper mantle. Both Press and Birch (*Phys. Earth Planet. Interiors*, **3**, 178; 1970) interpreted these high densities to favour almost exclusively an eclogitic composition for the upper mantle, and thus for the low velocity zone, although Birch did add that an iron-rich olivine upper mantle might still be possible. At this point, however, it became clear that any detailed discussion of an eclogitic upper mantle would be seriously hampered by the relative lack of experimental data on eclogites, in particular on their elastic properties.

Some results on the seismic velocities in eclogites have now been provided by Rao *et al.* (*Earth planet. Sci. Lett.*, **23**, 15; 1974), and they show just how involved the situation is. Eclogites from India, Czechoslovakia and Japan all have comparable garnet compositions and mean atomic weights, but there are significant differences in P wave velocities and velocity-pressure gradients. Velocities at 4 kbar, for example, range from 7.43 km s^{-1} for the Indian eclogites, through 8.05 km s^{-1} for those from Japan, to 8.35 km s^{-1} for the Czechoslovakian samples. For the same location order, average velocity-pressure gradients are, respectively, 0.08, 0.058 and $0.036 \text{ km s}^{-1} \text{ kbar}^{-1}$.

The reason for these variations is that the apparent distribution constant (*K*) of Fe–Mg between coexisting garnets and pyroxenes varies from one set of eclogites to the other, indicating that the conditions of temperature and pressure under which they formed were different. In fact, Rao and his colleagues are able to demonstrate a linear relationship between P wave velocity and *K*. But details apart, the general point to be made is that if the eclogite model is now to be considered seriously, much more experimental work will be required to elucidate the complexities of eclogite systems—as much, perhaps, as has been done on the more popular pyrolite systems.

Erratum

In the article "The Linear Differentiation of Chromosomes" (*Nature*, **252**, 95; 1974) the statement was made that "the many current attempts to explain banding reflect our lack of knowledge of how 1 cm of DNA can be packed with protein into a 1 mm transverse band". 1 cm of DNA is in fact packed into a $1 \mu\text{m}$ transverse band.

review article

Congenitally abnormal vision in Siamese cats

R. W. Guillery, V. A. Casagrande & M. D. Oberdorfer

In albino mammals, the absence of pigmentation in the eye is associated with abnormalities of the visual pathway and these result in a scrambled input to the visual centres of the brain. Recent research on Siamese cats, in which more than one pattern of abnormality has been found, shows that there are several ways in which the cortex can deal with the abnormal input, and is beginning to define the rules according to which the cortex selects its inputs.

THE sensory centres of the brain carry an orderly representation of the environment as shown by many topographically organised sensory 'maps'. While it seems likely that such orderly representations are essential for the proper functioning of sensory mechanisms, it is difficult to test this rather general view, because one cannot easily challenge sensory mechanisms by providing them with a scrambled input. Recently, however, the discovery that some mammals have congenitally abnormal visual pathways has provided an opportunity for testing how cerebral mechanisms can respond to disordered inputs, and studies of the abnormal pathways have begun to show some of the rules on the basis of which cerebral representations of the outside world can be established.

Mammals with abnormally low amounts of pigment in the retinal pigment epithelium have abnormal pathways linking the eyes to the brain¹⁻⁸. Generally, the abnormality involves nerve fibres that arise in the temporal parts of the retina. Whereas in normal animals all such fibres would pass to the cerebral hemispheres without crossing (Fig. 1), in the abnormal animals some of the temporal fibres cross the midline (Fig. 2a).

Retino-geniculate connections in Siamese cats

The precise pattern of the abnormal projection has been worked out in most detail for the lateral geniculate nucleus of Siamese cats⁹⁻¹¹, and Fig. 2(a) summarises the results obtained by anatomical and electrophysiological methods⁹. The basic pattern of the abnormality is simple. Some of the retino-geniculate axons go to the wrong side of the brain, but the geniculate locus of all axon terminals relates to the retinal locus of their cells of origin in a normal mapping. Thus, in Fig. 2a, segments 10*, 11* and 12* of the left retina are shown passing to the right lateral geniculate nucleus instead of the left. Within the right nucleus they terminate in the appropriate cell layers (shown as layer A1 in Fig. 2) and the axons that arise in segment 10* end medially, while those arising in segment 12* end laterally, as is normal. It can be seen that segments 5*, 6* and 7* from the left eye, which would normally map opposite segments 5⁺, 6⁺ and 7⁺ from the right eye, in Siamese cats map opposite segments 12*, 11* and 10*, respectively, from the left eye.

The distribution of the abnormal fibres does not vary greatly in Siamese cats. There is always a lateral, normally connected segment of lamina A1 (3⁺ and 4⁺ in Fig. 2a, the 'lateral normal segment') and next to it is a large

abnormally connected segment, the 'lateral abnormal segment'. The border between the two (arrow in Fig. 2a) generally receives from retina that lies 15° to 20° from the line of decussation. Most of the cats that have been examined also show a small 'medial normal segment' of lamina A1 (8⁺ in Fig. 2a), but the size of this segment varies somewhat between animals, and it may possibly not occur at all in some Siamese cats (see below).

Fig. 2a shows that the lateral geniculate nucleus receives an abnormal representation of the visual field. It seems as though there are geniculate mechanisms that sort axons in terms of retinal topographies, but that there are no mechanisms for correcting the nonsense appearing in terms of orderly visual field representations. We shall see that this is in striking contrast to the situation in the visual cortex.

There are three major consequences of the simple retinogeniculate misrouting, and we shall refer to them as a 'disruption', an 'inversion' and a 'non-correspondence'. The first term refers only to the fact that within one geniculate layer the visual field segments are represented in an interrupted manner. An inversion occurs when a portion of the visual field representation is a reversal of the normal, so that one finds an ascending sequence in one part of layer A1 (10*, 11*, 12* in Fig. 2a) and descending sequence in another portion of the same layer (4*, 3*). An inversion is a special case of a disruption, and each involves only a single geniculate layer. A non-correspondence, which is any mismatching of two adjacent layers, is necessarily an interlaminar phenomenon. It is possible to produce a non-correspondence without a disruption and to produce a disruption without a reversal, and thus, it is useful to consider the three as distinct abnormalities.

The geniculo-cortical pathways in Siamese cats

Figure 1 shows that in a normal cat the visual cortex receives an orderly, binocular representation of the contralateral visual field. Hubel and Wiesel¹² have established that most neurones in the visual cortex receive inputs from both sets of geniculate layers and can be activated through either eye. Such a balanced, binocular geniculo-cortical projection would clearly lead to serious problems in Siamese cats, since cortical activity would provide an ambiguous representation of the environment. Figure 2b shows that if the geniculo-cortical pathways in Siamese cats were arranged as in normal cats, some cortical cell groups would be activated by two quite different parts of the visual field. Further, a movement from left to right across the visual field would be represented ambiguously

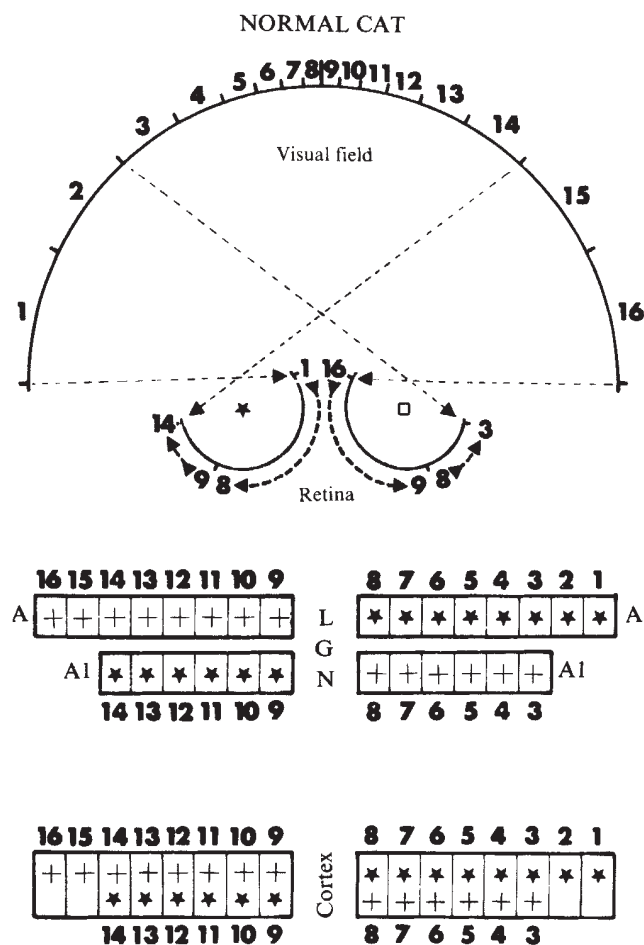


Fig. 1 The normal retino-geniculo-cortical projections in a cat. The representations of the visual field within the visual centres are shown as numbered sequences. Those coming through the right eye are marked by plus signs, and those coming through the left eye by stars.

within the cortex, being in one direction for segments 5*, 6* and 7*, and in the opposite direction for segments 10*, 11* and 12*.

Let us first consider the non-correspondence produced by the abnormality in terms of information available about normal cats. The visual cortex of normal cats can be made to receive noncorrespondent inputs by quite simple manipulations. When normal cats are reared with a surgically induced squint, the inputs to adjacent geniculate layers are made non-correspondent and single cortical cells no longer receive binocularly matching stimuli¹³. The geniculo-cortical pathway in such cats becomes modified so that individual cortical cells receive a monocular input. Cells receiving from each eye tend to be grouped together in distinct cortical columns. Within the cortex, instead of a single binocular representation of the visual field, two separate monocular representations are formed, and these are arranged as a mosaic of interdigitating monocular columns. A similar result has been obtained in kittens reared with alternating occlusion of the eyes¹³. Again, cortical cells were exposed either to right eye or left eye stimulation; since they were never exposed to correspondent binocular inputs, no binocular cortical columns were formed.

On the basis of these observations on non-correspondent geniculate inputs one might expect a similar result in Siamese cats. Considering first a Siamese cat with no strabismus, one might expect the situation illustrated in Fig. 2b, which shows a hypothetical, not an actual cortical

pattern. Binocular columns are shown in the cortical regions that receive from the normal segments of the lateral geniculate nucleus (3, 4, 8 in Fig. 2b) and a mosaic of monocular columns is shown for the abnormal segments (5*, 6*, 7*, 10*, 11*, 12*). A cat with a significant strabismus, and this is quite common in Siamese cats, might be expected to show two independent sets of monocular columns for the whole visual field representation. The Siamese cats that have been studied so far, however, have shown a strikingly different cortical pattern.

Cortical suppression of the temporal visual field

The pattern of neuronal activity that we have found in the visual cortex of most Siamese cats¹⁴ is shown schematically in Fig. 3a. The input coming through lamina A from the contralateral retina (1* to 8*) appears to be quite normal, but it is difficult to record much cortical activity corresponding to an input from lamina A1. Segments 8*, 10* to 12*, 4* and 3* are, therefore, shown as thinner figures and smaller symbols in Fig. 3a. It seems that the visual cortex in these animals is looking at the whole visual field through the A laminae on each side, and that all of the inputs coming through the A1 laminae, which contain an inversion, are suppressed.

The cortical activity suggests that these Siamese cats have a normal field of view when they are using both eyes, but when they are only using one eye the effective field of view is limited to the parts of the visual field seen by the nasal retina. That is, the cat sees on the side of the open eye, but does not see across the midline at all.

Elekessy *et al.*¹⁵ have used behavioural methods to study the visual fields of Siamese cats. They showed, independently, almost the exact limitation of the visual fields that our results led us to expect. Whereas normal cats have about 135° of visual field in each eye^{16,17} (Fig. 1), their Siamese cats had only about 90° of visual field extending from close to the midline to the temporal periphery (1 to 8 for the left eye in Fig. 1).

It is clear that these Siamese cats do not develop a binocular mosaic of independent monocular inputs. Instead, there is massive suppression of the inverted segment (10*, 11*, 12*, in Fig. 3a) and even the segments of the visual projection that are normal (3*, 4*, 8*, in Fig. 3a) do not establish a demonstrable, cortical field. The suppression in these normally connected segments shows that lateral interaction must play a part in the development of the geniculo-cortical pathways. Segments 3* and 4* in Figs

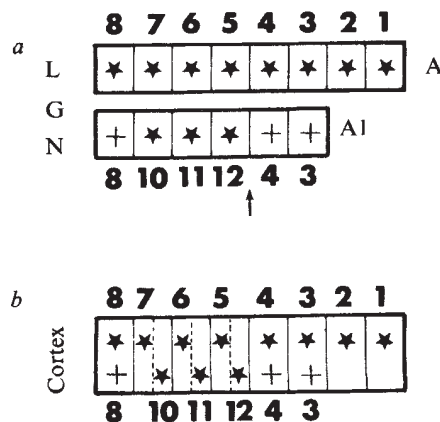


Fig. 2 The abnormal geniculo-cortical pathway to the right hemisphere in Siamese cats. *a*, Abnormal representation of the visual fields found in the right lateral geniculate nucleus of Siamese cats. *b*, Cortical patterns that might be expected if the geniculo-cortical pathways were normal (see text).

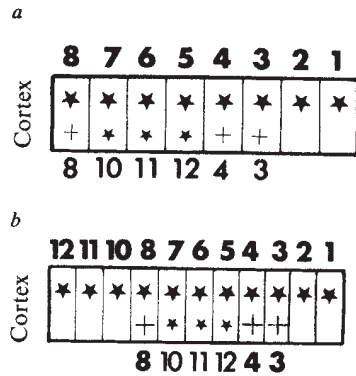


Fig. 3 *a*, Cortical representations of the visual field found in 'Midwestern' Siamese cats. *b*, Cortical representations of the visual field found in 'Boston' Siamese cats. The lighter numbers and smaller symbols represent a weak cortical input.

2 and 3a can only be regarded as abnormal because they form a part of a sequence that contains an inversion. It seems that the cortex does not accept inverted representations of the visual field, even though it can accept non-correspondent ones.

Correction of the inverted representation

While the cortical pattern shown in Fig. 3a is found in some Siamese cats, other Siamese cats show a second pattern. This has been demonstrated electrophysiologically by Hubel and Wiesel¹¹ and by our own anatomical evidence¹⁴. The most striking feature of this second pattern is the reversal of the geniculo-cortical projection of segments 10*, 11* and 12* (see upper row in Fig. 3b) and the insertion of these segments into cortex that normally receives from close to the zero vertical meridian. The cortical input from these abnormal geniculate segments to the expected cortical locus (opposite 5*, 6* and 7*) is correspondingly reduced. The total amount of the visual cortex is not increased in these cats, but about 135° of visual field is represented in the cortex instead of the normal 90°.

The sequence of the visual field representations shown in bold figures is normal, except for the hiatus between 8 and 10, and the size of this hiatus on the right corresponds to the size of the medial normal geniculate segment on the left (9* and 9*). Thus, the modified geniculo-cortical projection in these cats has corrected the reversal, but appears to have left a small disruption. Any cat in which the medial normal segment was small or absent would show a virtually complete recreation of the normal sequence of visual field segments, and it is possible that the size of the medial normal segment is critical in determining whether the cortical pattern shown in Fig. 3a or that shown in Fig. 3b will be formed. If normal cortical function depends upon an uninterrupted sequential ordering of the visual field representations in the cortex, a cat with a large medial normal segment, which could establish a normal sequence only by suppressing the input from layer A1, would have to use the solution shown in Fig. 3a, while a cat with a small medial normal segment might use the more elegant solution illustrated by Fig. 3b.

We have called these two types of cortical pattern the Midwestern (Fig. 3a) and the Boston pattern (Fig. 3b)¹⁴. We do not know whether the formation of one or the other is related to the size of the medial normal segment. In fact, we know nothing of the mechanisms controlling the development of these pathways. The problem has been discussed by Gaze and Keating¹⁸, and it is relevant that the solution they propose for the formation of the Boston pattern also assumes an absence of the medial normal segment.

No one has yet tested the visual fields of a Boston cat. From the cortical records that have been obtained¹¹, however, one can reasonably expect that Boston cats have a normal 135° field of view. The visual field test may therefore prove to be useful for further studies of the Siamese abnormality, since it is relatively simple and can be made without any injury to the cat. Some such test for distinguishing Boston from Midwestern cats will be needed for studies of the inheritance patterns of the abnormalities and for further developmental studies.

Effects of monocular deprivation in Siamese cats

The effect of the inversion in the lateral geniculate nucleus can be tested by raising Siamese cats with one eye sutured. In Midwestern cats monocular deprivation will have the effect shown by crosses in Fig. 4. On both sides the inversion of the visual field representation within lamina A1 will be abolished. Further, the parts of lamina A1 receiving visual input will form a continuous, though partial representation of the visual field on one side (5*, 6*, 7*), and a disrupted representation on the other (3*, 4*, 8*). This disruption should be distinguished from the type of disruption that occurs, for example, in Fig. 3b between segments 8 and 10. In Fig. 3b there are no geniculate segments between 8 and 10, and we regard this as a 'strong' disruption. In Fig. 4 the amount of geniculate nucleus between segments 4 and 8 is appropriate for the intervening visual field segments, and we call this a 'weak' disruption.

In normal cats monocular deprivation upsets a balanced binocular competition and leads to a dramatic reduction in the cortical input from the deprived eye^{17,19,20}. In Siamese cats monocular deprivation will upset this balance in geniculate segments 3, 4, 8 (Fig. 2a), but will not affect it in the rest of the nucleus.

Three Siamese cats that were raised with one eye sutured have been studied electrophysiologically¹⁴. All three cats showed a Midwestern pattern, but in each, stimulation of the temporal retina activated cortical cells more effectively than in normal Siamese cats: although the input from lamina A was still dominant throughout the cortex in these three kittens, all parts of lamina A1, except for the normal deprived segments (9*, 13*, 14*) showed this increased effectiveness. These changes occurred within the abnormal segments even though the balance of the trans-laminar interaction had not been altered at all: it was the sequence of the representation within lamina A1 itself that had been altered by the deprivation.

More recently we have raised three Siamese kittens with one eye sutured from postnatal day 9 until they were 6–9 months old. We then tested their visual fields behaviourally, using essentially the same methods as Elekessy *et al.* (see ref. 17 for details). Visual responses were tested along guidelines at 15° intervals and we found that all three kittens had about 135° of visual field for the nondeprived eye (Table 1 and Fig. 5). That is, the visual fields of the nondeprived eye were like those of a normal cat, as would be expected from our earlier electrophysiological observations. Suturing one eye had increased the effective visual field of the other.

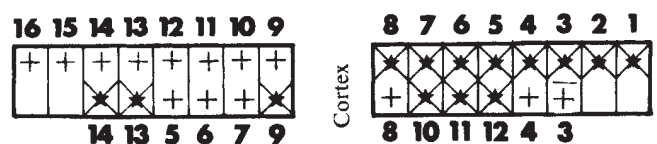


Fig. 4 Schema to show the expected effects of monocular visual deprivation in a Midwestern Siamese cat. Segments that are deprived by suturing the left eye shortly after birth are shown by large crosses.

Table 1 Test scores for guidelines at 15° intervals. (% of correct responses: 50 trials for each score)*

Animal	Eye tested	Degrees from midline							Notes
		On side of open eye			On side of closed eye				
668	R	105	90	75 to 0	15	30	45	60	
	L	0	28	80-100	51	0	0	0	
OM	R	0	37	80-100	26	0	0	0	
	L	11	73	90-100	50	6	0	0	
OF	R	10	55	90-100	92	14	0	0	
	L	21	84	100	33	0	0	0	
636	R	7	69	90-100	73	24	0	0	Left eye sutured
	L	0	56	90-100	95	98	54	24	
637	R	15	86	90-100	100	98	36	0	Right eye sutured
635	L	17	85	90-100	96	85	19	0	Right eye sutured
635	R & L	0	43	100	100	83	83	0	Sutures removed at 7 months

* Final scores are corrected as follows: Where blank scores (B) were greater than guideline scores (G) (see ref. 17 for definition) the corrected scores were 0. Where $G > B$ the corrected score was $\frac{G-B}{100-B}$

We regard all three kittens as Midwestern: the retrograde degeneration produced by a cortical lesion has demonstrated this for one of the kittens (see ref. 14 for method). The other two are still living, but since the overwhelming majority of the cats we have obtained locally have been Midwestern cats, and since the two parents (OM and OF) and one unrelated control (668) have shown a limitation of the monocular fields that is not normal (Table 1 and Fig. 5), the conclusion that we were dealing with Midwestern cats seems justified, even though there is some spread of visual responses across the midline in the two parents. This spread may represent uncontrolled eye movements or may, perhaps, indicate some vision in the temporal retina. Since the amount of the visual sparing is distinctly less than would be expected in Boston cats and since there are no grounds for expecting such a limited sparing in Midwestern cats, we favour the former view.

It remains to be pointed out that in these three kittens we were testing cortical, not tectal functions: it is known that the tectal visual fields demonstrable behaviourally do not cross the midline (ref. 16 and M. S. Sherman, unpublished), and the behavioural results matched the activity recorded in cortical nerve cells of comparable cats¹⁴.

The visual cortex in the monocularly deprived Siamese cats was, therefore, able to produce accurate control of movement in spite of the fact that within one area of cortex two different parts of the visual field were represented in reverse order (10°, 11°, 12°; 7°, 6°, 5°). The non-correspondence was dealt with successfully, even though the two representations were opposed in direction, and presumably a mosaic of alternating monocular columns was established in the cortex. The critical difference between the normal and the monocularly deprived Siamese cats is the lack of any inversion within a single geniculate layer. Thus, the cortex must monitor its geniculate inputs in terms of the sequences coming from individual layers. Non-correspondences can be accepted, even when they involve complete reversals, but inversions within one geniculate layer are unacceptable. The weak form of the disruption occurring between segments 8° and 5° in Fig. 4 seems not to prevent the formation of cortical connections, but we have seen that there is some indirect evidence that the strong form of the disruption occurring between segments 8° and 10° in Fig. 3b may be unacceptable.

When the tests summarised above were completed, the sutured eye was opened and the testing continued with this eye only. To date, we have found no signs of any vision in the deprived eye. This is in marked contrast to a normal

cat: when a normal cat is raised with the left eye sutured, the visual functions are spared in the right monocular geniculo-cortical segments (1 and 2 in Fig. 1) within which the deprived pathway has not had to compete with the normally innervated one. Even though segments 1 to 4 are

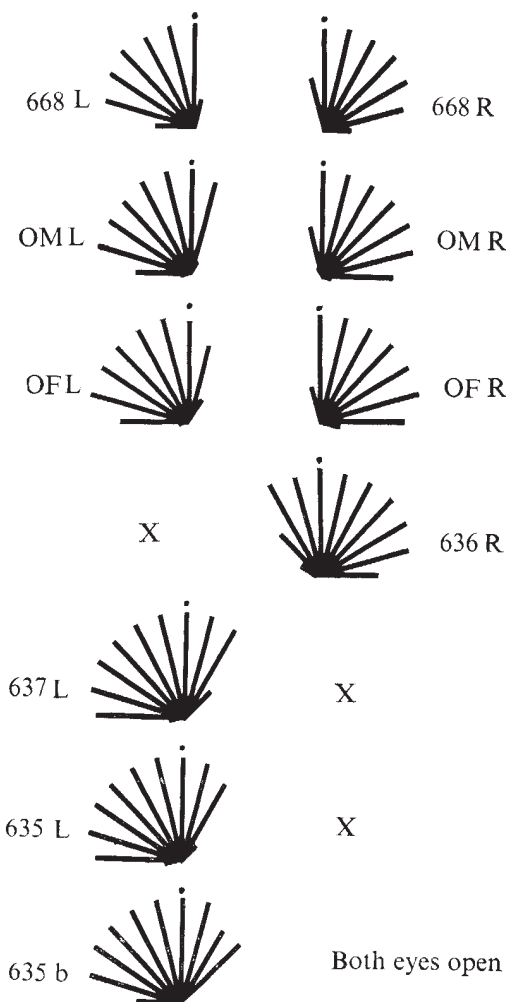


Fig. 5 Schematic representation of corrected guideline scores from table 1. The midline is indicated by a dot. X shows the sutured eye. 635b shows visual fields in a cat that was raised with the right eye sutured and that was subsequently tested with both eyes open.

connected into the retinocortical pathways in the same way in normal and Siamese cats, segments 1 and 2 react to lid suture in quite different ways in the two sorts of cat.

This is a puzzling observation and there is, at present, no direct evidence to explain it. We suggest that the difference must be produced by the abnormality in segments 10*, 11* and 12* (Fig. 4). If an adult cat is no longer able to establish a mosaic of alternating monocular columns, then the cortex of our monocularly deprived cats had no way to separate the lamina A inputs from the lamina A1 inputs when the deprived eye was opened. The total new input pattern contained an inversion and this is why the whole sequence was suppressed at cortical levels.

It would seem then that the development of cortical 'maps' depends upon the coherence of the sensory representation that can be established in the cortex. A non-correspondence can be turned into two independent coherent cortical representations in an infant, but probably not in an adult. Incoherence, whether produced by a strong disruption, by an inversion, or by a fusion of non-correspondent inputs, leads to blocked development of a cortical representation. In contrast to this, geniculate representations, even when they are incoherent in terms of the visual field, are successfully established on the basis of retinal locus.

We have shown that the abnormality found in albino mammals can be used to study the mechanisms by which orderly visual field representations may be established. So far we have only considered problems that arise in the geniculo-striate system of Siamese cats. Other parts of the visual pathway are also abnormal in these cats^{10,21,22}, however, and it may prove that they develop according to different rules. Further, the precise pattern of the abnormality differs in other species. Albino ferrets show a very large abnormality and have practically no uncrossed retinogeniculate axons⁴. Abnormal pathways have been found in several genotypes of mink⁸ and the size of the abnormality, which is related to the amount of pigment in the retina, varies considerably. Thus there is a variety of models on which one can test hypotheses about the visual pathways.

So far our studies of Siamese cats have shown that in at least one sensory system the cortex monitors the sequence of its sensory inputs, and that there are definable rules according to which disrupted inputs can be rejected.

We thank Dr J. H. Kaas who collaborated in many experiments and Mrs E. Langer and Mrs B. Yelk who helped with the preparation of materials. The work was supported by grants from the Public Health Service, National Institutes of Health.

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articles

Relationships between sealevels, ¹⁸O variations and orbital perturbations, during the past 250,000 years

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Sealevel changes over the past 250,000 yr, determined from dated coral reefs on rising islands, are synchronous with precessional changes in solar radiation when orbital eccentricity is large. Oxygen isotope variations in deep-sea cores correspond in timing but not in amplitude, reflecting changing isotopic composition of Pleistocene icecaps.

WORLD climates during Quaternary times were influenced by two primary factors. First, there have been variations in the geographical and seasonal distribution of solar radiation, which resulted from perturbations in the Earth's orbital motion. Second, there was the role of the oceans in transferring heat from low to high latitudes, thus influencing strongly the periodic growth of the Laurentide and Fennoscandian Pleistocene icecaps.

The analysis by Milankovitch¹ of orbital perturbations

and variations of radiation has been supported by several workers²⁻⁴. This astronomical theory of climatic change has, however, been criticised⁵⁻⁸, principally because changes of radiation are themselves very much smaller in magnitude than the observed Quaternary temperature changes. Advocates of the astronomical theory suggest that icecap growth and decay may both be self-amplifying processes, under certain conditions, and that triggering from one state to the other is effected by the changing distribution of solar radiation^{9,10}. The case for the astronomical theory rests essentially on a correspondence between insolation curves and well dated records of Quaternary temperatures or ice volumes. Until recently, oxygen isotope and micro-palaeontologic studies of deep-sea cores provided the best basis for such arguments. The statistical methods for correlation between the core records and insolation curves have, however, been disputed^{11,12}; and the time scales for the cores are also disputed, with consequent disagreements about correlations¹³⁻¹⁷.

Results which lend support to the astronomical hypothesis come from flights of coral reef terraces that occur in rapidly rising areas, and which have been dated by ²³⁰Th/²³⁴U. The important results are from Barbados^{4,18,19}, New Guinea²⁰⁻²², and the Ryukyu Islands^{23,24}. Age estimates for Upper Quaternary sealevel maxima agree closely between the areas²², and from New Guinea the record is especially good. The recurrence of transgression-regression cycles with a period of about 20,000 yr has been claimed to reflect the precession effect upon Pleistocene climates^{4,20}, and the New Guinea sealevel curve correlates strongly with insolation changes over the last 0.23 Myr (ref. 25). Figure 1 shows sea level level changes during that period, together with $\delta^{18}\text{O}$ records from deep-sea cores from the Caribbean²⁶, and the equatorial Pacific²⁷. Orbital perturbation effects for the same period are also shown. To allay previous disputes about time scales and palaeosealevels, these curves will be discussed more fully.

The sealevel curve is based on the series of transgression-regression cycles identified from successions of offlapping coral reefs, mainly in New Guinea, which have been dated precisely by ²³⁰Th/²³⁴U with supporting ¹⁴C data²⁰⁻²². The estimated magnitudes of sealevel oscillations, and the basis for separating tectonic and eustatic effects have been explained²⁰⁻²². The curve for the past 0.14 Myr is taken directly from the most recent results, and this is highly in agreement with the less complete Barbados record²². The curve before 0.14 Myr is an updated version of that published earlier^{20,21}, based on precise levelling and field observations which I made in 1973, and which will be published elsewhere. The only significant difference between the new and the earlier curve is that the regression between 0.17 Myr and 0.14 Myr achieved a level of at least -100 m and possibly lower (as against the previous estimate of -55 m or lower). The $\delta^{18}\text{O}$ curves shown are from Caribbean core P6304-8 (ref. 26) and equatorial Pacific core V28-838 (ref. 27); both are based on the pelagic foram *Globigerinoides sacculifera*. The time scale for the cores was given by Shackleton and Opdyke²⁷, and was based on clear identification of the Brunhes-Matuyama magnetic boundary, and the assumption of constant sedimentation rate. This time scale is very similar to that proposed by Broecker *et al.*^{14,17}, and is about 25% longer than that proposed by Emiliani^{16,26}. The critical issue of a time scale for the cores is subject to a significant scatter in reported U-series age determinations, and opinions diverge about core dating by this method¹³⁻¹⁵. To find additional support for the shorter time scale, Emiliani *et al.* argued that the generalised palaeotemperature curve shows good peak correspondence with the 65°N isolation curve³, and with dated raised beaches¹⁶. Many statistical arguments of this kind are, however, incorrectly founded²⁵, and there is no significant correspondence between the New Guinea sea-

level curve and Emiliani's generalised palaeotemperature curve²⁵. I concur²⁵, however, with the results of Kemp and Eger²⁸, which support a significant correspondence between Emiliani's curves and the high latitude insolation curve. The correlation is not as strong as those evident in Fig. 1, however, and this presentation of the most accurate sealevel and $\delta^{18}\text{O}$ results to date is taken as resolving the time scale argument for deep-sea cores in favour of the Broecker-Shackleton-Opdyke scale.

The most important features of Fig. 1 are first, the large number of peak-to-peak correspondences, especially between the sealevel curve and the precession-dominated curve of variations of Earth-Sun distance; second, the small degree of comparability between sealevel and $\delta^{18}\text{O}$ curves in terms of relative amplitudes of oscillations; and third, the greater excursions of $\delta^{18}\text{O}$ shown in the Caribbean core relative to the Pacific core (true also for other Atlantic and Caribbean cores²⁹). These facts have extensive implications for ice age mechanisms. The very strong correspondence between fluctuations in sealevel and variations of the mean solar distance shows that the growth and decay of high latitude icesheets is influenced very strongly by insolation at low latitudes (that is because precession effects dominate insolation curves at low latitudes; obliquity dominates at high latitudes).

Control of Quaternary climatic fluctuations by variations in insolation at low latitudes was suggested by Broecker^{4,30}: it is to be expected on meteorologic grounds³¹; and the principle is reinforced by the finding³² that radiation input at high latitudes is quite inadequate to have caused Late Wisconsin deglaciation in the time available.

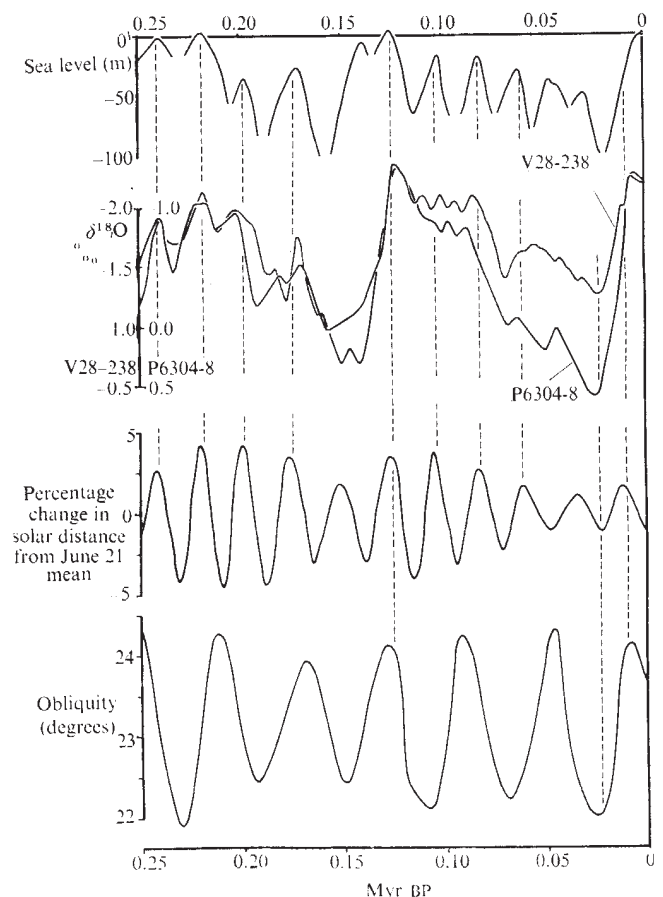


Fig. 1 Sea levels, $\delta^{18}\text{O}$ variations, and orbital perturbation effects for the last 250,000 yr. Emiliani's results²⁶ for core P6304-8 are plotted on the time scale of Shackleton and Opdyke²⁷ (see text). P6304-8, Atlantic core²⁶; V28-238, Pacific core²⁷.

The Laurentide and Fennoscandian ice sheets—over 85% of the Pleistocene ice excess⁸—were nourished principally from the North Atlantic^{8,33}, into which the Gulf Stream–North Atlantic current is the major conveyor of heat.

The relative amplitude differences between sealevel and $\delta^{18}\text{O}$ curves are important, as the latter have often been argued to be directly indicative of the former^{34–36}. The greatest discrepancy is between 0.115 Myr and 0.08 Myr, when two sealevel oscillations of about 50 m occurred: from the $\delta^{18}\text{O}$ record, Shackleton²⁷ has inferred approximately constant temperatures and sea levels, fluctuating within a 15 m range. From 0.075 Myr to 0.015 Myr the amplitudes of sealevel oscillations are again underestimated using $\delta^{18}\text{O}$. There is also an important shift in the mean $\delta^{18}\text{O}$ level. For the Pacific core, $\delta^{18}\text{O}$ is about +0.25‰ with respect to the mean Holocene level, from 0.115–0.08 Myr; from 0.075–0.015 Myr the shift is about +0.75‰. The sealevel curve shows that considerable icecaps waxed and waned from 0.12–0.08 Myr BP, thus $\delta^{18}\text{O}$ evidence suggests either that mean temperatures were considerably higher during this period than in the next 0.065 Myr, or that the ice sheets formed around 0.11 Myr BP and 0.09 Myr BP were not as relatively poor in ^{18}O as those from 0.075 Myr BP to 0.015 Myr BP. The latter hypothesis may be simply evaluated. Mean sealevel between 0.115 Myr BP and 0.08 Myr BP (about 1% of mean ocean depth). Mean ocean enrichment of $\delta^{18}\text{O}$ was $\approx 0.25\text{‰}$ and therefore ice formed should show $\delta^{18}\text{O}$ impoverishment by -25‰ . For the period 0.075–0.015 Myr BP was about -75 m , and with mean ocean $\delta^{18}\text{O} \approx 0.75$, ice formed in that period would show $\delta^{18}\text{O} \approx -37\text{‰}$. Such a $\delta^{18}\text{O}$ shift pattern is evident in the Greenland ice core³⁷. These estimates indicate the general result of ocean volume change, and are not intended to negate palaeotemperature inferences (the effect of which the uptake of ^{18}O by marine organisms, has been well demonstrated³⁸).

If the amplitudes of oscillations in the $\delta^{18}\text{O}$ curves are low compared with the sealevel curve, that can be explained partly by mixing in the sea-bottom sediment²⁷, and by the vertical migration of pelagic forams in response to glacial–interglacial salinity changes³⁹. It is also partly explicable in terms of the $\delta^{18}\text{O}$ distribution in precipitation over the icecaps. Precipitation at the centre and northern sector of the Laurentide icecap is likely to have been impoverished by about 20‰, relative to the southern margin⁴⁰. During icecap retreat, the water returned to the ocean was mainly from the periphery and the southern sector until the retreat was well established, whilst snow continued to fall over the icecap centre. This snow may have been more than 10‰ depleted in ^{18}O , with respect to the meltwater. Although deep ice flow would move highly ^{18}O -deficient ice from the centre outwards, the rate would be slow compared with wastage of the flanks^{40,41}. Thus the mean $\delta^{18}\text{O}$ level should rise during icecap retreat (up to a certain stage of icecap diminution), and the ocean $\delta^{18}\text{O}$ swing is therefore reduced relative to the sealevel swing. Estimation of the magnitude of this 'fractionation' effect requires careful modelling.

There are two possible explanations for the apparent shift about 0.08 Myr ago in the mean $\delta^{18}\text{O}$ value of icecap-nourishing snow. A change of climate may have occurred, so that the moisture-bearing airmasses which originated over warmer oceans before 0.08 Myr BP, originated over cooler oceans subsequently. Alternatively, the icecaps around 0.112 Myr BP and 0.095 Myr BP may have been nearly as extensive at the later (Main Wisconsin) sheets, but only about half as thick. These alternatives are deduced from the pattern of $\delta^{18}\text{O}$ values over high northern latitudes, reported by Dansgaard and Tauber⁴⁰. The ocean temperature effect is illustrated by the $\delta^{18}\text{O}$ difference between precipitation over water fed by the Gulf Stream at 60°N in the Norwegian Sea (-7‰) and at the same latitude in

the Labrador Basin (about -15‰). The mean surface water temperature difference is about 6°C. The altitudinal effect is indicated by an impoverishment of up to 20‰ in the precipitation over central Greenland, relative to the coasts (a height difference of about 2.5 km).

The $\delta^{18}\text{O}$ shift of about -10‰ around 0.08–0.07 Myr BP in the ice core from Camp Century, Greenland³⁷, suggests that there was a simultaneous alteration of high latitude climate. This is because Greenland ice is very unlikely to have been 1 km thicker than at present during main Wisconsin times, for dynamical reasons (1 km is approximately the additional elevation needed to produce the observed $\delta^{18}\text{O}$ shift by the altitude effect). The altitude effect cannot be ruled out for the inferred Laurentide and Fennoscandian icecaps of around 112 Myr BP and 95 Myr BP, as these were not dominated by an ocean boundary; however, a climatic change influencing Greenland would have affected these also. The ocean temperature effect on $\delta^{18}\text{O}$ levels in snowfall seems to be implicated. The warmer sources of ice-nourishing precipitation 0.12–0.08 Myr BP were either the northern Atlantic or the Arctic Oceans. For the $\delta^{18}\text{O}$ effect to have stemmed from the Arctic, the Arctic Ocean must have been open water, a condition likely to be stable⁴² even with a moderate ice cover on the encircling land masses. Open water in the Arctic, suggested by Ewing and Donn^{43,44} as a likely factor in initiating glaciation, would lead to mean Arctic surface water temperatures up to 9°C higher⁴². Arctic deep-sea cores indicate an ice cover on the sea over the last 70 Myr (refs 45 and 46), but interpretations differ for the core record before that time. The evidence from core T3-67/11 is that foram productivity was much greater from about 0.13–0.07 Myr BP than in either the preceding or succeeding 0.07 Myr (ref. 45). Such heightened productivity is consistent with open water in the Arctic^{45,46}. Detailed analysis of further cores is necessary. It may be that the record from the Norwegian Sea, which Kellogg⁴⁷ amply demonstrated to have been considerably more ice-bound than at present during the last 0.12 Myr indicates sufficiently that the Arctic is unlikely to have been ice free. The conditions of the ocean surface in the northern Atlantic must therefore have altered substantially between 0.08 Myr and 0.07 Myr ago, with probably a fall of temperature and perhaps a major expansion of ice cover over the sea.

Rather rapid alterations of regional climate, occurring well after glaciation is established, may thus be responsible for important differences between $\delta^{18}\text{O}$ and sealevel records for the last 250,000 yr. Within glacial epochs, icecaps oscillated in size with about a 20,000-yr period. When orbital eccentricity is large, icecap growth and decay is synchronous with precession-induced variations of mean solar distance at the summer solstice. These facts must be incorporated in any adequate theory of ice ages.

Received April 9; revised July 3, 1974.

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Statistical geometrical approach to random packing density of equal spheres

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A statistical geometrical argument is presented which could, as the basis of a more refined calculation, help to give a statistical geometrical answer to the question of what random packing is.

'WHAT is random packing?' That question has been much discussed since random packings of spheres were first proposed as models of simple liquid structure¹⁻⁶. Experimental determinations of the maximum packing density agree on a value of about 0.636 ± 0.001 (refs 7-16), but there is still no satisfactory statistical geometrical theory to explain this value, though a rough explanation based on coordination number distribution has been given by one of us¹⁷. Moreover, the lower limit of stability of a random packing occurs at a density of about 0.60 (refs 9-13); no explanation of this 'random loose packing' figure exists. We present here a statistical geometrical argument which derives a packing density of 0.6357 in excellent agreement with the recognised experimental values. We also obtain a lower stability bound at a density of 0.6099, which we suggest corresponds to the random loose-packing.

The statistical argument

Our argument is threefold:

(1) As a consequence of a stability criterion, the average coordination number in the packing should be 6.0.

(2) As first pointed out by Bernal⁵, the random packing can be thought of as a collection of tetrahedral aggregates of spheres. We calculate the most probable shape of the characteristic tetrahedron.

(3) We then deduce the overall packing density from that of the most probable tetrahedron.

Average coordination number

For a given sphere to be stable against displacement in a given direction, it must be supported by three spheres in that direction, the group of four spheres forming a tetrahedron. The same stability criterion must also apply for the opposite direction, implying thus a coordination number average of 6. This proposition was examined by Heppes¹⁸ for a serially-deposited packing in which no rearrangements are permitted. Bennett¹⁹ later argued that the same should be true of a packing further densified by shaking or otherwise. Figure 1 is a plot of the average coordination number against radial distance for the full Finney 7934 centre close-packed model²⁰ and shows an average of 6 (allowing for experimental errors in the sphere coordinates). Each point is the average value of 500 to 700 central spheres and full curve is the data line of Mason²¹. A recent study²² on a simulated very slow settling of rigid equal spheres into a randomly packed bed gives also an average coordination number of 6.0, with an overall packing density of 0.58.

The most probable tetrahedron

Consider a tetrahedron formed by an arbitrary sphere and three supporting spheres. We postulate that the three supporting spheres supporting a central sphere are oriented according to a probability that is proportional to that projected surface area of the spherical envelope at $R = 1.0$ diameters which can be seen from a long distance from the supported sphere. We ignore the possible restricting effect of the neighbours on the configuration of the three supporting spheres.

In the coordinate system shown in Fig. 2a, we calculate the expected z coordinate of a first supporting sphere. The elementary annular surface area of the spherical envelope at $z = z_1$ which can be seen from distant z direction is

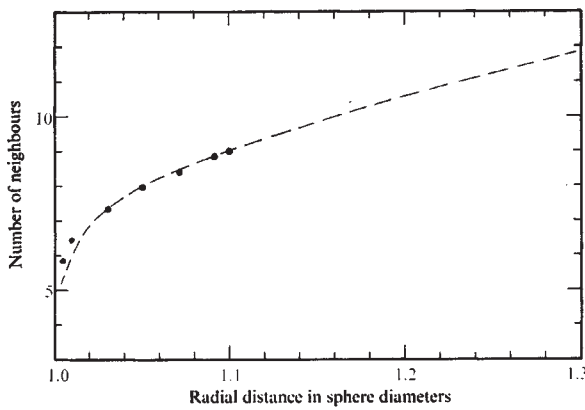


Fig. 1 The average number of neighbouring spheres within the radial distance R in units of the sphere diameter. ●, this experiment; ---, ref. 21.

$$dS = 2\pi r_1 \cdot d\phi_1 \sin \phi_1 = 2\pi z_1 dz_1$$

where the subscript 1 denotes the first supporting sphere. The probability that the z coordinate of the first supporting sphere lies between z_1 and $z_1 + dz_1$ becomes

$$f(z_1) dz_1 = (2\pi z_1 dz_1) / \left(\int_0^1 2\pi z_1 dz_1 \right) = 2z_1 dz_1 \quad (1)$$

where $f(z_1)$ is the probability density function, and the denominator in the middle term of equation (1) expresses the total area available for the first sphere. From equation (1) the expected value of z_1 becomes

$$\bar{z}_1 = \int_0^1 z_1 \cdot f(z_1) dz_1 = 2/3 \quad (2)$$

where our units are sphere diameters. $\bar{z}_1$ is the largest of the z coordinates of the three supporting spheres.

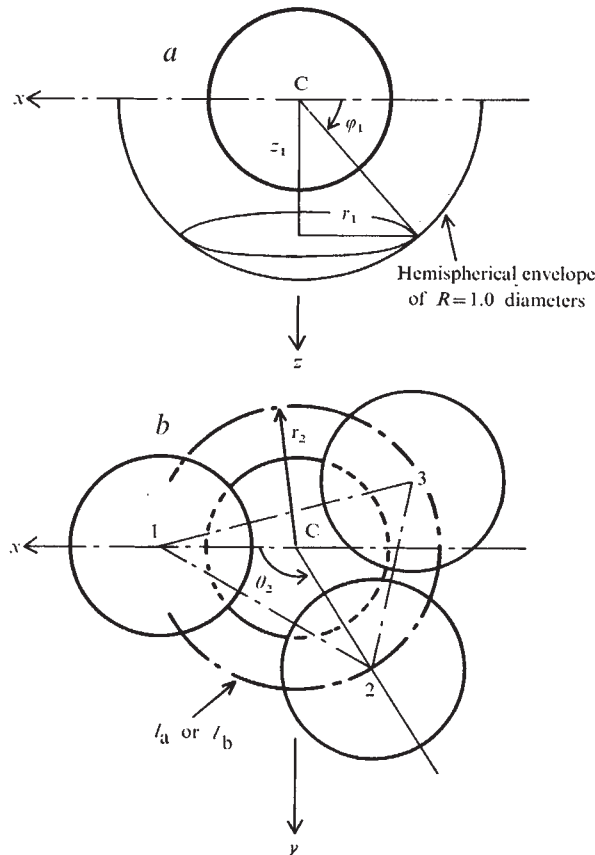


Fig. 2 The coordinate system. C denotes the central sphere; 1, 2, 3, the supporting spheres; l_a or l_b , the length used in equation (3).

We can partially test our assumptions and argument so far with reference to the 7934 centre packing. We first define the 'shortest zig-zag path' between the arbitrary spheres S and E (Fig. 3). Around the sphere S and on the side of the sphere E we search sphere centres lying within the hemispherical volume of radius R . We simultaneously obtain the distance D_i from each sphere centre to the line SE and also the SE-directional

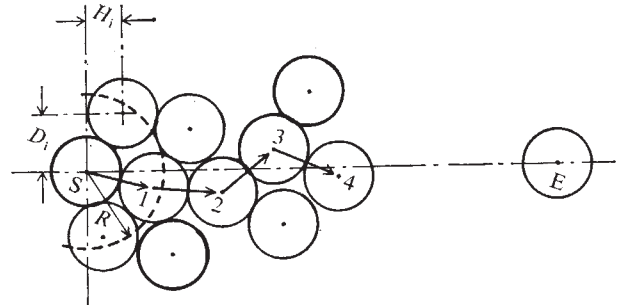


Fig. 3 The shortest zig-zag path.

component H_i of the distance from the sphere S to each sphere centre. Then we move from the sphere S to the next central sphere 1 which lies nearest to the line SE (that sphere for which D_i is minimum). The procedure is repeated until we reach the sphere E. In this way we have obtained information on the packing structure along with the length of the shortest zig-zag path between two arbitrary spheres. The first supporting sphere as defined above lies on the shortest zig-zag path, and equation (1) expresses the H_i distribution of the shortest zig-zag path, which agrees on average with the experimental result fairly well (Fig. 4).

For ease of later calculation, we now fix the first supporting sphere at the point $(\sqrt{5}/3, 0, 2/3)$ —we place the sphere on the xz -plane. Our final result is independent of this choice provided $\bar{z}_1 = 2/3$.

Subject to the existence of the first supporting sphere, we now calculate the expected z coordinate of a second supporting sphere. The projected surface area of the spherical envelope which can be seen from distant z direction becomes (Fig. 2b)

$$\begin{aligned} S_2 &= \int_{z_2=0}^{z_2=2/3} l_a \sin \phi_2 d\phi_2 + \int_{z_2=2/3}^{z_2=z_*} l_b \sin \phi_2 d\phi_2 \\ &= \int_0^{2/3} [(l_a z_2 dz_2) / \sqrt{(1-z_2^2)}] + \int_{2/3}^{z_*} [(l_b z_2 dz_2) / \sqrt{(1-z_2^2)}] \\ &= \int_{\sqrt{5}/3}^1 l_a dr_2 + \int_{r_*}^{\sqrt{5}/3} l_b dr_2 \end{aligned} \quad (3)$$

where

$$\begin{aligned} l_a &= 2 \int_{-r_2}^A [(r_2 dx_2) / \sqrt{(r_2^2 - x_2^2)}] \\ &= 2r_2 \left[\sin^{-1} \frac{(5/9) - (1-r_2^2)}{2r_2 \sqrt{5/3}} + \frac{\pi}{2} \right] \end{aligned}$$

with $A = (5/9 - z_2^2) / (2\sqrt{5/3})$

$$\begin{aligned} \text{and } l_b &= 2 \int_{-r_2}^B (r_2 dx_2 / \sqrt{(r_2^2 - x_2^2)}) \\ &= 2r_2 \left[\sin^{-1} \frac{(1-4\sqrt{(1-r_2^2)/3})}{2r_2 \sqrt{5/3}} + \frac{\pi}{2} \right] \end{aligned}$$

with $B = (1-4z_2/3) / (2\sqrt{5/3})$

and $r_* = \sqrt{(1-z_*^2)} z_* = (2+\sqrt{15})/6$

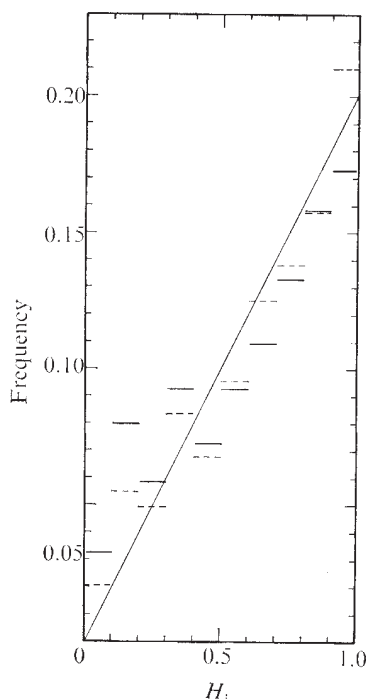


Fig. 4 The H_i distribution for the spheres on the shortest zig-zag paths. Full line, Equation (1); — and — — —, experiments for $R = 1.005$ and $R = 1.01$, respectively.

The l_a term is obtained for the restrictions $(x_2 - \sqrt{5/3})^2 + y_2^2 \geq 1$ and $z_2 \leq 2/3$; the l_b term for $(x_2 - \sqrt{5/3})^2 + y_2^2 + (z_2 - 2/3)^2 \geq 1$ and $z_2 \geq 2/3$. Using equation (3), the expected z value of the second supporting sphere becomes

$$\bar{z}_2 = \left(\int_{\sqrt{5/3}}^1 z_2 l_a dr_2 + \int_{r_*}^{\sqrt{5/3}} z_2 l_b dr_2 \right) / S_2 = 0.61154365 \text{ diameters} \quad (4)$$

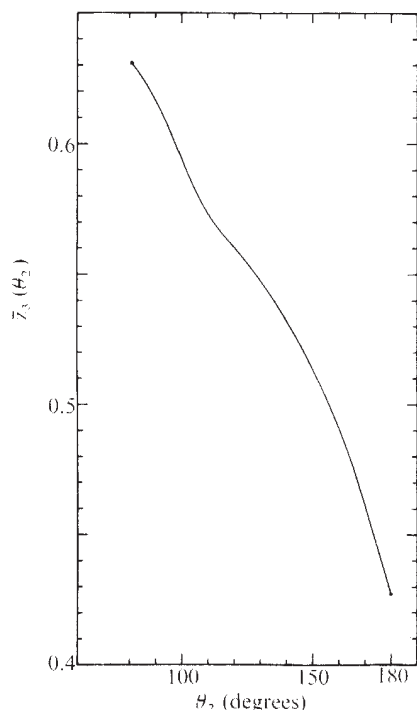


Fig. 5 $\bar{z}_3(\theta_2)/\theta_2$ relation.

where $z_2 = \sqrt{1 - r_2^2}$. Although $\bar{z}_2$ has been obtained, expected values of x_2 and y_2 remain unknown. We now express the coordinates of the second supporting sphere as $(r_2 \cos \theta_2, r_2 \sin \theta_2, 0.61154365)$, where $r_2 = \sqrt{1 - \bar{z}_2^2}$ (see Fig. 2b).

For a given value of θ_2 we can now calculate the projected surface area S_3 of the spherical envelope on which the centre of a third supporting sphere lies, subject to the following conditions: (1) the centre of the third supporting sphere lies on the surface of the spherical envelope and we require the projected area which can be seen from distant z direction; (2) the three supporting spheres do not intersect amongst themselves— $d_{13} \geq 1$ and $d_{23} \geq 1$ (where d_{ij} denotes the distance between the two sphere centres, i and j); and (3) the triangle consisting of the centres of the three supporting spheres, which can be seen from distant z direction, must include the projected centre of the central sphere (Fig. 2b). This is the stability condition for the central sphere. As is seen from Fig. 2b, we need consider only one side of xz plane, and sphere 2 touches sphere 1 at $\theta_2 = 80.995^\circ$, giving the range of $80.995^\circ \leq \theta_2 \leq 180^\circ$. On the way to calculating numerically the projected surface area S_3 , we can simultaneously obtain the expected value $\bar{z}_3(\theta_2)$ of the third supporting sphere for $\theta = \theta_2$. In the numerical calculation, minute surface areas satisfying the above three conditions were summed up for a fixed z value. In this way we obtain the $\theta_2/\bar{z}_3(\theta_2)$ relation and its mathematical mean value becomes

$$\bar{z}_3 = \int_{80.995^\circ}^{180^\circ} \bar{z}_3(\theta_2) d\theta_2 / (180^\circ - 80.995^\circ) = 0.54093 \quad (5)$$

$\bar{z}_3(\theta_2)$ decreases monotonically with increasing θ_2 (Fig. 5); we find $\bar{z}_3 = 0.54093$ corresponds to $\theta_2 = 134.53^\circ$. Thus we have determined the expected coordinates of the second supporting sphere. We now express the expected coordinates of the

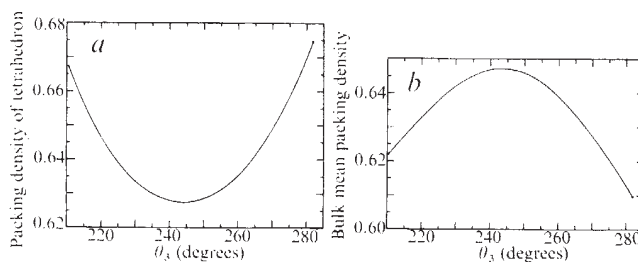


Fig. 6 *a*, Probable packing density of the tetrahedron. *b*, Probable density of the random packing.

third supporting sphere as $(r_3 \cos \theta_3, r_3 \sin \theta_3, 0.54093)$, where $r_3 = \sqrt{1 - \bar{z}_3^2}$, and sphere 3 touches sphere 2 and sphere 1 at $\theta_3 = 210.44^\circ$ and 281.93° , respectively, giving a possible range of $210.44^\circ \leq \theta_3 \leq 281.93^\circ$.

Next we consider the tetrahedron consisting of the centres of the central sphere and the three supporting spheres. The packing density Ψ of the spheres in the tetrahedron can be expressed by a function of θ_3 , the Ψ/θ_3 curve being U-shaped with upper bounds of about 0.667 and 0.675 with a minimum of 0.627 (Fig. 6a). The position of the third supporting sphere, in other words θ_3 , is still unknown at this stage.

The formulae used in the above calculation are collected in ref. 23.

Overall packing density

So far we have discussed only the most probable shape of the tetrahedron, a collection of which constitutes the random packing. As the tetrahedron cannot be considered to be a 'unit cell' of the packing, its density will not necessarily correspond to the bulk density. Instead, we refer to Finney's polyhedral analysis¹⁶ in which the packing of spheres is considered in terms of the packing of the 'Voronoi polyhedron' or

'Dirichlet region' (refs 24, 25), and relate part of the most probable tetrahedron discussed above to the most probable Voronoi polyhedron. We postulate that this average polyhedron is characteristic of the packing, and thus gives us the bulk packing density.

First we bisect perpendicularly the distances between the central sphere and the three supporting spheres by three planes, dividing the tetrahedron into two parts. The inner part, the central sphere side, is considered as a representative angular segment of the Voronoi polyhedron confining the central sphere, since the most probable tetrahedron is discussed for an arbitrary direction and the system must be isotropic overall. Thus we can calculate the packing density of the angular segment, that is the bulk mean density Ψ_{bulk} of the random packing. As is shown in Fig. 6b, the $\Psi_{\text{bulk}}/\theta_3$ curve is convex with lower bounds of 0.6099 and 0.6220, with a maximum of 0.6472, and a mathematical mean value of 0.6357. Since no criterion is available for determining θ_3 , we suggest the mathematical mean value is the expected bulk mean density of the random packing; namely, that the random packing density of equal spheres is 0.6357 (with possible bounds of 0.6099 and 0.6472), in excellent agreement with the recognised experimental maximum value of 0.636 ± 0.001 (refs 7-16). In addition, the stability criterion implies upper and lower density bounds of 0.6472 and 0.6099. The latter we suggest might be identified with the random loose packing.

Validity and application of the technique

Our argument is not rigorous, but its consistency with experiment is encouraging. In particular, we stress the following problems: (a) the average coordination of 6 is still open to argument, although the experimental data support it, even for the close-packed aggregate; (b) we have ignored the restrictive effects of neighbouring configurations, and so probably underestimated cooperative geometrical effects; (c) we have prematurely averaged our distributions. More elaborate calculations could avoid this objection, and would presumably give a $\theta_3 : \Psi$ distribution having a slightly different shape and bounds.

Thus we must be wary of assigning too much significance to the actual values obtained. The mean value 0.6357 is temptingly close to the accepted experimental maximum density values of Scott^{1,2} and of Finney¹⁶ (0.6366 ± 0.0008 and 0.6366 ± 0.0004 respectively) but we cannot assert definitely that the mean value

should necessarily correspond to that maximum density obtained in the laboratory. We might rather suggest that the laboratory maximum density should correspond to our upper bound of 0.6472, while the lower bound of 0.6099 should refer to the minimum packing density for an isotropic stable packing—the so-called random loose-packing. If this assignment is correct, both bounds are about 0.01 too high, a discrepancy which may be due to our admittedly premature averaging; alternatively, both the Scott and Finney packings may not have been true maxima.

This work was started at Birkbeck College. K.G. thanks the Japanese Ministry of Education for a visiting Research Fellowship.

Received April 29; revised July 5, 1974.

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Structural uniqueness of lactose operator

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The repressor binding capacity of λ plac DNA was maximally reduced by as few as 2-5 cuts by either of two single-strand specific nucleases. But approximately 300 cuts by three nonspecific nicking agents were necessary to give the same effect.

THE properties and conformation of DNA are dictated by nucleotide sequence^{1,2}. This conclusion was derived from at least fourteen different chemical, physical, enzymatic and biological studies on twelve high molecular weight duplex DNAs with defined repeating nucleotide sequences. The effect of sequence on structure was apparent with these DNA polymers since the influence was magnified compared with natural DNAs whose properties are a composite of many types of sequence effects.

We are extending these studies by attempting to determine if

natural DNA has the same conformation throughout its entire length or if different structures exist at various sites along the chain. It is presumed that regions with 'anomalous' conformations are present in small amounts compared with the bulk DNA (which may be in a typical DNA B configuration). Thus, a highly sensitive and specific assay is needed to detect regions with unique conformations. Also, if these odd regions are present in small amounts, they may serve as regulatory sites, instead of structural genes. Their uniqueness may contribute to the high degree of specificity and tightness of binding of regulatory proteins.

The *lac* repressor plus λ plac DNA system³ provides a highly sensitive and selective assay for a specific region of DNA, since the repressor binds to operator DNA with a remarkably large equilibrium constant⁴ and does not perform complex catalytic or translocating functions. Nucleases with defined specificities were used as selective probes for assessing the

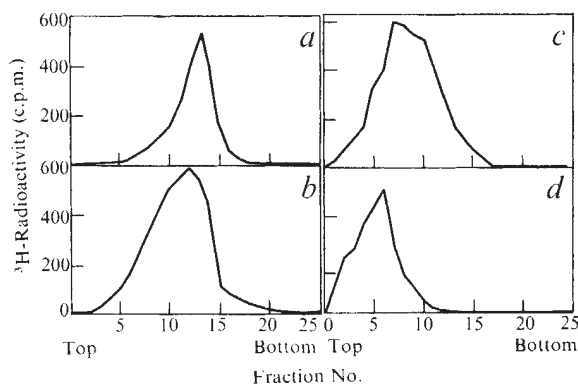


Fig. 1 Alkaline sucrose gradient profiles of ^3H - λ -*plac* DNA treated with S_1 nuclease. For preparation of λ -*plac* 5 phage, medium J (ref. 14) was used but with $10 \mu\text{g ml}^{-1}$ of thymidine and 0.4% of glucose instead of lactose. ^{32}P and ^3H -thymidine-labelled λ -*plac* 5 DNA was prepared from strain JG 108 λ -*plac* (provided by A. Riggs). A 100 ml culture was grown at 30°C to an A of 0.4 and induced at 45°C for 15 min. 2 mCi of ^{32}P -phosphate or ^3H -thymidine was added on induction and the culture was shifted to 37°C and incubated for an additional 3 h. Cells were centrifuged, resuspended in 10 ml of $\Phi 80$ buffer (50 mM NaCl, 1 mM MgSO_4 , 20 mM Tris-HCl, pH 7.4) and lysed with chloroform. After centrifugation, the phage were concentrated with polyethylene glycol 6,000. The precipitated phage were centrifuged and resuspended in 5 ml of $\Phi 80$ buffer. Solid CsCl (optical grade) was added to a final density of 1.49 g ml^{-1} and this suspension was centrifuged for 14 h at 35,000 r.p.m. in a Spinco Ti-50 rotor. Phage, purified from two cycles of CsCl gradients, were dialysed against DB I (10 mM Tris-HCl, pH 7.4, 10 mM MgSO_4). The DNA was extracted from the phage by rolling the solution with an equal volume of phenol six times and the excess phenol was removed by six ether extractions. The aqueous layer was dialysed exhaustively against and subsequently stored in DB II (2 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). Immediately before use, DNA stored in DB II was dialysed against DB III (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). All dialysis tubing was autoclaved and stored in sterile buffer before use. DNA prepared by this procedure contained few, if any, detectable single-strand scissions (a). S_1 nuclease was purified to 23 units mg^{-1} by the procedure of Vogt⁶. Reactions with S_1 nuclease contained 10 mM ammonium acetate (pH 5.0), 1 mM ZnSO_4 , 50 mM NaCl, $15 \mu\text{g ml}^{-1}$ of ^3H - λ -*plac* DNA and variable amounts of S_1 nuclease. We define one unit of S_1 nuclease as the amount of enzyme required to render acid soluble¹ 25 nmol of ^3H -(dA-dT)_n (dA-dT)_n per min at 37°C in a 100 μl reaction mixture containing 650 nmol of ^3H -(dA-dT)_n (dA-dT)_n instead of λ -*plac* DNA. Reactions (0.10 ml) were performed at 37°C for 30 min and were terminated by addition of EDTA (to 25 mM). The reaction products were extracted three times with phenol and six times with ether. Recovery was greater than 90%. These samples were subsequently used for molecular weight determinations or repressor binding studies. Linear 5 to 20% (w/v) alkaline sucrose gradients were made in 0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA. DNA samples (50 μl) were layered on to the gradients and were centrifuged at 45,000 r.p.m. for 2.5 h at 4°C in an SW 50.1 rotor and the radioactivity of 0.2 ml fractions was determined. Molecular weights of the DNA fragments were calculated relative to markers of intact λ -DNA and Φ X174 DNA (from J. Dodgson). a, No S_1 nuclease; b, 0.1 units nuclease; c, 1 unit nuclease; d, 10 units nuclease. Fraction 13, 15×10^6 ; fraction 10, 7×10^6 ; fraction 7, 3×10^6 and fraction 5, 1×10^6 .

structural uniqueness of the operator DNA. The comparative ability of two single-strand specific nucleases (mung bean and S_1 nuclease) and of three nonspecific nicking agents (pancreatic DNase, micrococcal nuclease and sonication) to abolish repressor binding ability as a function of reduction in DNA molecular weight was determined.

Single-strand specific nucleases

S_1 nuclease cleaves double-stranded λ -*plac* DNA into large fragments even though it has an absolute preference for single-stranded over double-stranded DNA^{5,6}. ^3H - λ -*plac* DNA was degraded by S_1 nuclease to different extents and the sizes of

the DNA fragments were determined on 5–20% alkaline sucrose gradients (Fig. 1). Under the conditions used, treatment with 1 and 10 units of nuclease caused an average molecular weight reduction from 30×10^6 (undegraded) to 6×10^6 and 4×10^6 , respectively. If the DNA were single stranded, the latter concentration of enzyme was sufficient quantitatively to degrade the DNA to acid-soluble fragments (results not shown).

Gel electrophoretic analyses were performed on 0.6% agarose gels on selected DNA fragments, which had been denatured, to provide a separate determination of molecular weights and to provide greater resolution. Figure 2 shows the distribution of S_1 nuclease-generated fragments and the distribution of molecular weight markers. Substantial agreement was found between the positions of the shoulders on the sucrose gradient analysis (Fig. 1) at fractions 5, 7 and 10 and the sizes of the predominant species on gel analysis (Fig. 2) at fractions 45, 37 and 30, representing molecular weights 1.2×10^6 , 3×10^6 and 7×10^6 respectively. Also, both analyses showed the virtual absence of undegraded DNA (15×10^6 , single-strand molecular weight) and of small fragments ($< 100,000$) consistent with the expected mode of action of S_1 nuclease^{5,6}. These analyses reinforce the validity of the size determinations. Also, the gel analyses suggest that the DNA was cut at specific sites since a nonrandom distribution of S_1 fragments was generated (compare with distribution of *Eco* R₁ fragments in Fig. 2).

The ability of the S_1 nuclease fragmented ^3H -DNA to compete against undegraded ^{32}P -DNA for *lac* repressor is shown in Fig. 3. The more highly degraded DNAs bind repressor less well.

Figure 4 correlates the data from the first three figures and shows that a small reduction in molecular weight caused by S_1 nuclease provides a large reduction in repressor binding activity. The binding was reduced 50% when the λ -*plac* DNA (molecular weight, 30×10^6) was degraded into fifths (molecular weight, 6×10^6). If this number of scissions occurred on a random basis, the repressor binding ability of the DNA should be unaltered ($< 1\%$ reduction).

Studies were performed also with the mung bean nuclease which prefers single-stranded over double-stranded DNA by a

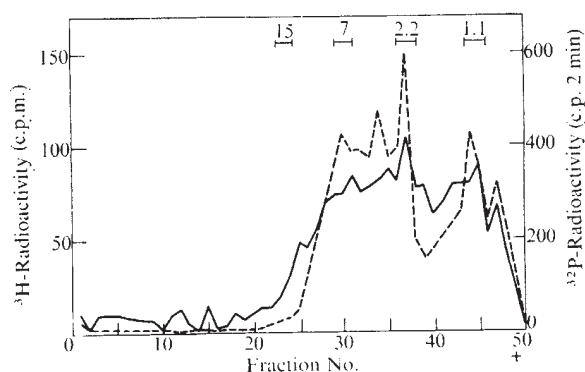


Fig. 2 Agarose gel electrophoresis of ^3H - λ -*plac* DNA treated with S_1 nuclease. (—), ^3H - λ -*plac* DNA treated with 1 unit of S_1 nuclease, as described in Fig. 1c; (---), fragments of ^{32}P - λ -*plac* DNA treated with the *Escherichia coli* R₁ restriction enzyme (from B. Weisblum). The *Eco*R₁ reaction mixture contained 100 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , $5.75 \mu\text{g ml}^{-1}$ ^{32}P - λ -*plac* DNA and sufficient enzyme to degrade all ^{32}P -DNA. The 50 μl reactions were incubated at 37°C for 15–30 min. ^3H -sample DNA and *Eco*R₁ restricted ^{32}P - λ -*plac* DNA markers were denatured with 0.2 volume of 2 M NaOH and were made 10% in sucrose and 0.04% in bromophenol blue. The mixture was electrophoresed for 5 h at 5 mA per gel at room temperature on 0.6% w/v agarose gels¹⁵, with continuous circulation of the electrophoresis buffer (36 mM Tris, 30 mM NaH_2PO_4 , 1 mM EDTA, 0.05% SDS (pH 7.7)) between the upper and lower chambers. Transverse 3 mm gel slices were dissolved in 1 ml water by autoclaving and radioactivity was determined. The 15×10^6 molecular weight marker was unfragmented, but denatured, λ -*plac* DNA. Solid line, ^3H ; broken line, ^{32}P .

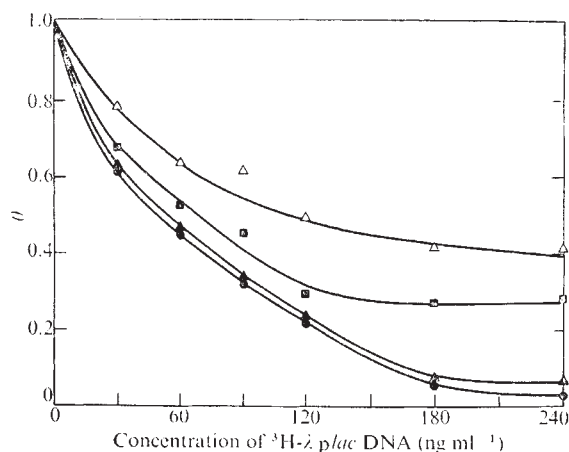


Fig. 3 Repressor binding ability of λ plac DNA treated with S_1 nuclease. ^3H - λ plac DNA was treated with variable amounts of S_1 nuclease (see Fig. 1) and the affinity of the nuclease-nicked ^3H - λ plac DNA for the lactose I^{80} repressor (provided by A. Riggs) was determined by competition equilibrium experiments as described¹. The operator active repressor was determined as described¹ and was at least 95% pure. Various amounts of test ^3H - λ plac DNA were mixed with 0.03 μg of intact ^{32}P - λ plac DNA in 0.5 ml of BB^{14} . After addition of repressor (1.2×10^{-15} mol), the mixture was incubated for at least 30 min at room temperature; 0.2 ml aliquots were then filtered in duplicate through nitrocellulose membranes (pretreated as described¹⁴). The membrane filters were washed once with 0.4 ml of FB^{14} , dried, and radioactivity was determined. Each experimental point is the average of two filters; reproducibility was $\pm 10\%$. $\blacktriangle$, No enzyme; $\bullet$, 0.1 units; $\blacksquare$, 1 unit; $\triangle$, 10 units of S_1 nuclease. The repressor binding ability θ , is the ability of the nuclease-treated ^3H -DNAs to compete against intact ^{32}P - λ plac DNA for the lactose repressor. θ is obtained by dividing the ^{32}P -counts retained in the presence of competing ^3H -DNA by that retained in the absence of competing DNA.

ratio of 30,000 to 1 (ref. 7). The mung bean nuclease is at least as effective as the S_1 nuclease (Fig. 4) in destroying repressor binding. When approximately two double-stranded cuts were made, which decreased the molecular weight by one-third (10×10^6), a 50% reduction of repressor binding was found. Further degradation of the DNA with more enzyme to a molecular weight of 0.5×10^6 gave only a slightly greater (60–70%) reduction in binding. The reason for the lack of total reduction in repressor binding by these two nucleases is unknown. That the mung bean enzyme is slightly more specific than the S_1 nuclease may be due to its higher extent of purification and hence single-strand specificity; trace contamination with a double-strand specific endonuclease could diminish substantially the specificity demonstrated in Fig. 4.

Nonspecific nicking agents

Since the S_1 and mung bean nucleases had a preferential effect on repressor binding sites, λ plac DNA fragmentations were performed with pancreatic DNase, micrococcal nuclease and sonication, agents which do not have pronounced specificities. λ plac DNA was degraded with pancreatic DNase over a 10^{10} fold concentration range. The size of the products was analysed by alkaline sucrose gradient sedimentation and ranged from approximately 15×10^6 to 100,000 (duplex molecular weights) (Fig. 4). Repressor binding activity of these fragments was substantial (greater than 85%) even when the DNA was fragmented to 3% (1×10^6) of its original size. To observe a 50% reduction of repressor binding ability, λ plac DNA had to be degraded into fragments no larger than 100,000 in molecular weight (Fig. 4).

As it was important to determine accurately the size of the sample most highly degraded by pancreatic DNase and since this sample remained near the meniscus on an alkaline sucrose gradient, indicating a molecular weight no larger than 100,000, a determination was performed on 10% polyacrylamide gels

under established conditions. The DNA was at least 160 base pairs long indicating a molecular weight of at least 100,000. Hence, the dual analyses set the molecular weight of this sample at 100,000.

Similar studies were carried out with two other nonspecific agents for degrading DNA which gave virtually identical results. Degradations were performed with micrococcal nuclease, which generates 3'-phosphoryl termini, and with ultrasonication, a random mechanical process. Reactions with micrococcal nuclease (Worthington) contained 50 mM Tris-HCl (pH 7.4), 2 mM CaCl_2 , 15 $\mu\text{g ml}^{-1}$ of ^3H - λ plac DNA and variable amounts of micrococcal nuclease. λ plac DNA was degraded into double-stranded fragments of approximately 15×10^6 , 3×10^6 and 0.5×10^6 by both techniques (results not shown). Repressor binding studies demonstrated that the 15×10^6 and the 3×10^6 molecular weight preparations had no detectable reduction in repressor binding activity and that the 0.5×10^6 preparations retained at least 80% of this activity. The results of these sonication studies are consistent with previous work⁸ which demonstrated that λ plac DNA could be sonicated into fragments of one million molecular weight and still retain full repressor binding.

Hence, the results from these three nonspecific fragmentation methods are internally consistent and emphasise the specificity of nicking by S_1 and mung bean nuclease (Fig. 4). Between 60 and 150 times as many cuts by the nonspecific agents are necessary to give the same extent of reduction in repressor binding as for the two single-strand specific enzymes.

Protection of operator by repressor

Since only a few nucleolytic scissions by the single-strand specific nucleases sufficed to diminish repressor binding ability effectively, the *lac* operator, or a nearby region, was the presumed site of nicking. To test this presumption, degradation

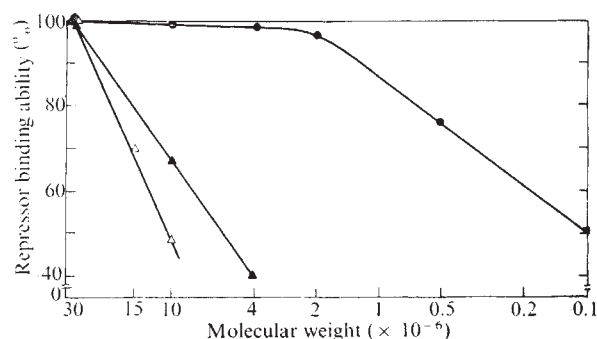


Fig. 4 Comparative ability of S_1 nuclease, mung bean nuclease, and pancreatic DNase to diminish repressor binding ability. S_1 nuclease studies were performed as described in Figs 1 and 3. Mung bean nuclease was purified at least 2,000-fold by a modification of the published method⁷. An accurate specific activity of the enzyme could not be determined since the highly purified enzyme contained no detectable protein. Reactions contained 10 mM ammonium acetate (pH 5.0), 15 $\mu\text{g ml}^{-1}$ ^3H - λ plac DNA and various amounts (0.1 to 10 units) of mung bean nuclease. Other details were described for S_1 nuclease. We define one unit of mung bean nuclease as the amount of enzyme that rendered acid soluble 65 nmol of ^3H -(dA-dT)_n·(dA-dT)_n per min at 37°C in a 100 μl reaction containing 650 nmol of ^3H -(dA-dT)_n·(dA-dT)_n and 10 mM ammonium acetate (pH 5.0). Reactions with pancreatic DNase (Worthington) contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 15 $\mu\text{g ml}^{-1}$ of ^3H - λ plac DNA and variable amounts (from 1×10^{-7} pg to 1 ng) of pancreatic DNase. Other details were described in Fig. 1. Molecular weight values determined under denaturing conditions were doubled. The percentage of repressor binding activity was obtained by dividing the quantity of intact ^3H - λ plac DNA required to obtain a θ value of 0.5 by the amount of the nicked ^3H -DNA to give the same value of θ . $\triangle$, Mung bean nuclease; $\blacktriangle$, S_1 nuclease; $\bullet$, pancreatic DNase; $\blacksquare$ molecular weight determined by both alkaline sucrose gradient and 10% polyacrylamide gel electrophoresis.

Table 1 Repressor binding ability of λ plac DNA treated with S_1 nuclease in absence or presence of repressor

Reaction	Additions	Molecular weight of DNA after treatment	Repressor binding %
1	None	30×10^6	100
2	S_1 nuclease	4×10^6	43
3	S_1 nuclease + repressor	4×10^6	>95
4	S_1 nuclease + denatured repressor	4×10^6	43

Reaction conditions were identical to those described in Fig. 1 except that no NaCl was present and 10 units of S_1 nuclease was used in all cases. 0.010 ml of repressor buffer (0.13 M potassium phosphate (pH 7.2) and 5% glucose) was added as the blank to reaction 2. Lactose repressor (threefold binding saturation excess at pH 7.0) was added to the DNA and incubated at 0° C for 15 min before the nuclease was added. Denatured repressor was obtained by heating the active repressor at 100° C for 10 min. Molecular weights and repressor binding capacities were measured as described in Figs 1 and 3.

studies were performed in the presence and absence of repressor (Table 1). With a constant amount of S_1 nuclease, the DNA was degraded to a similar extent (molecular weight, 4×10^6) in the absence or presence of repressor. This result was not surprising since the repressor should protect only a small portion of DNA (about 27 base pairs⁹). But when the ability of the fragmented DNAs to bind repressor was compared, substantial differences were found between fragments that were generated in the presence or absence of active repressor. A 60% reduction in repressor binding was observed when the DNA was degraded in the absence of added repressor. In contrast, when the nuclease degradation was performed in the presence of active repressor, more than 95% of repressor binding ability was retained. Also, heat-denatured repressor did not protect since the same amount of reduction in repressor binding was found when S_1 nuclease degradation was performed in the presence of heat-denatured repressor as in its absence. Similar experiments with the mung bean nuclease gave a result in complete agreement with the data of Table 1.

The significance of this protection is twofold. Reduction

of repressor binding by single-stranded nuclease treatment must be due to nucleolytic scission at sites identical or closely adjacent to the repressor binding site. Second, the recognition of the operator by the single-strand specific nucleases is not due to some artefactual conformation induced by the nuclease reaction conditions since the repressor binds specifically under the nuclease reaction conditions.

Effect of salt

The 'structural uniqueness' of the *lac* operator could be due to (a) a high degree of thermolability (facile breathing), (b) a unique but nonlinear structure containing some unpaired nucleotides such as a Gierer loop¹⁰ (cruciform) or (c) another unique conformation which is linear and fully base paired but has a different helical pitch or tilt of base pairs, such as found for certain DNA polymers^{1,2}. High salt concentrations should diminish the local breathing of thermolabile regions of DNA (theory a) and should diminish the tendency to form cruciforms (theory b) (in the absence of cruciform stabilising factors such as modified bases or proteins), but should have little or no effect on the tendency to adopt other unique but linear structures.

Comparative studies were performed with the S_1 nuclease in 50 and in 250 mM NaCl (results not shown). Since the S_1 nuclease was half as active in 250 mM as in 50 mM NaCl on a single-stranded substrate, three times as much nuclease was added to the high salt reactions to assure that the salt effect was solely on DNA conformation rather than the enzyme activity. Under both conditions, the λ plac DNA was fragmented to similar extents and the repressor binding ability of the treated DNA was similarly abolished; 70% reduction of repressor binding was found when the DNA was degraded in 250 mM NaCl to 1.4×10^6 (compare with Fig. 4).

Models for DNA Structure

The original goal of this study was to determine if different DNA conformations exist along a chromosome chain. Our results demonstrate that certain regions of λ plac DNA are quite sensitive to single-strand specific nucleases and, therefore, have structures that are different from the greater part of the DNA. This conclusion agrees with and was predicted from the results on DNA polymers^{1,2} which revealed the influence of nucleotide sequence on DNA properties and structure.

The nature of the structural uniqueness which is recognised by the S_1 and mung bean nucleases is unknown at present. Three possible models (Fig. 5) are envisaged: model a, the unique region possesses low thermostability and, hence, is in a nonhelical state for a larger proportion of the time than other regions of DNA. The low thermostability could be due to low G-C content or to the presence of modified nucleotides. Model b, the unique region could exist, at least transiently, in a cruciform structure¹⁰ or other configuration which contains some nonpaired bases. Model c, the unique region may be totally helical but in a different geometric configuration (not DNA B) than the great part of the DNA chromosome. For example, the tilt of the base pairs or the helical dimensions could be somewhat altered; such helical variances have been recognised^{1,2} with the DNA polymers. Whatever this odd configuration may be, it must be susceptible to the single-strand specific nucleases.

The nucleotide sequence of transcripts of the *lac* promoter and operator region has been determined (ref. 9 and Dickson, Abelson, Barnes, and Reznikoff, personal communication). Though revealing several interesting sequence anomalies, these studies do not provide direct information on the conformation of these regulatory sites. Two facets of the sequence may however be relevant to this work; the potential for the formation of a cruciform exists at the presumed repressor binding site. In a run of 11 possible base pairs, 10 are true Watson-Crick pairs. Additionally, several regions of high A-T content exist

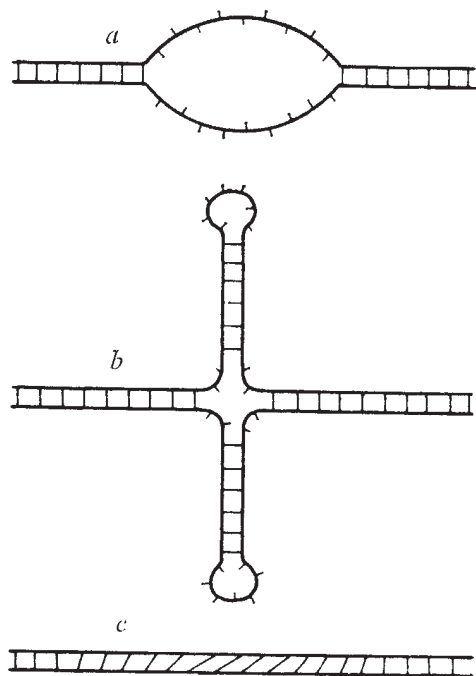


Fig. 5 Possible unique structures. a, Thermolability; b, cruciform; c, helical, non-DNA B.

in the promoter-operator sequence. The longest contains 10 A-T pairs in a run of 12 base pairs. The significance of these regions remains to be determined.

It is not possible to distinguish between these three models on the basis of present information. The nucleolytic sensitivity of the repressor binding activity of λ plac DNA was insensitive to salt concentration (see above) which strongly favours model *c* and disfavors *a* and *b* since the parent linear duplex structures should be stabilised by salt. Also, there is little effect of temperature on the binding of repressor to λ plac DNA⁴ which favours model *c* but disfavors *a* and *b*. Again, the large forward rate constant of the repressor-operator interaction⁴ makes probable the interaction of the repressor with a stable, not transient, unique operator configuration; this consideration favours model *c* and disfavors *a* and *b*. Model *a* is favoured by the presence of A-T-rich regions in the sequence and this model easily explains the results described here. The *lac* repressor-operator interaction is, however, quite sensitive to ionic strength⁴ which favours this model, but the sensitivity may be explicable on the basis of protein-protein, rather than protein-nucleic acid, interactions. Also, previous studies¹¹ with DNAs of defined sequence demonstrated the need for both high A-T content and certain sequence features for effective repressor binding; low thermostability alone was not sufficient.

The cruciform model (*b*) is plausible because of its simplicity and because of the sequence studies but is argued against by (1) its thermodynamic improbability and (2) recent studies¹² which demonstrate that λ plac DNA containing a high density of negative supercoil twists binds repressor only slightly better than non-supercoiled DNA. Negative supercoils should stabilise cruciform structures.

The extent of 'single strandedness' which is necessary to provide the results described here is unknown also but may be only a few nucleotides. Our results should not be interpreted as necessitating long stretches of random coil structure.

We anticipate that the nucleolytic scissions by the single-strand specific nucleases are at the repressor binding site, as implicated by the repressor protection experiment (Table 1). But it may be that a neighbouring region is the site of attack and that the influence is transferred along the DNA chain.

Experimental justification for the transmission of conformational stability (telestability) has recently been found (Burd, Wartell, and R.D.W. unpublished) with the duplex block polymer $d(C_{15}A_{15}) \cdot d(T_{15}G_{15})$.

That the *lac* operator may possess elements of single strandedness is consistent with the high degree of specificity of binding of the repressor since nonhelical DNA could provide substantially more characteristic sites for binding than helical DNA¹³.

This work was supported by the National Science Foundation. We thank Professor J. Dahlberg for determining the size of a DNA sample on polyacrylamide gels.

Note added in proof: Preliminary results show that the half-life for the S_1 nuclease nicked DNA-repressor complex is reduced from 20 min to approximately 10 min, thus explaining the lack of total abolition of repressor binding.

Received June 4; revised August 5, 1974.

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letters to nature

QSOs of high redshift?

We have completed a series of observations at Jodrell Bank on the radio structures of a complete sample of QSO candidates identified from the Parkes $\pm 4^\circ$ equatorial survey. Four sources are of particular interest in that they have properties which lead us to suggest that they may be QSOs of unusually high redshift.

At present we are not aware of any QSOs with steep radio spectra which have redshifts greater than 2.4; all those with higher redshifts have flat or concave radio spectra. (We use the spectral categories defined by Stannard¹, except that the spectral index is determined at 1 GHz observed frequency

rather than 3 GHz emitted frequency.) It is of interest to consider the properties that QSOs with steep radio spectra and high redshifts may possess. In common with most of the known high redshift QSOs they would probably lack an ultraviolet excess². A further property may be deduced from studies of angular diameter^{3,4} which suggest that, at high redshift, steep spectrum QSOs are likely to be compact, with angular diameters of less than a few arc s. Most of the flat and concave spectrum QSOs of high redshift have visual magnitudes between 18 mag and 19 mag. The studies of Setti and Woltjer⁵ and Stannard¹ suggest, however, that any steep spectrum QSOs of comparable redshift will be, on average, about one magnitude fainter. One reason for the apparent lack of steep

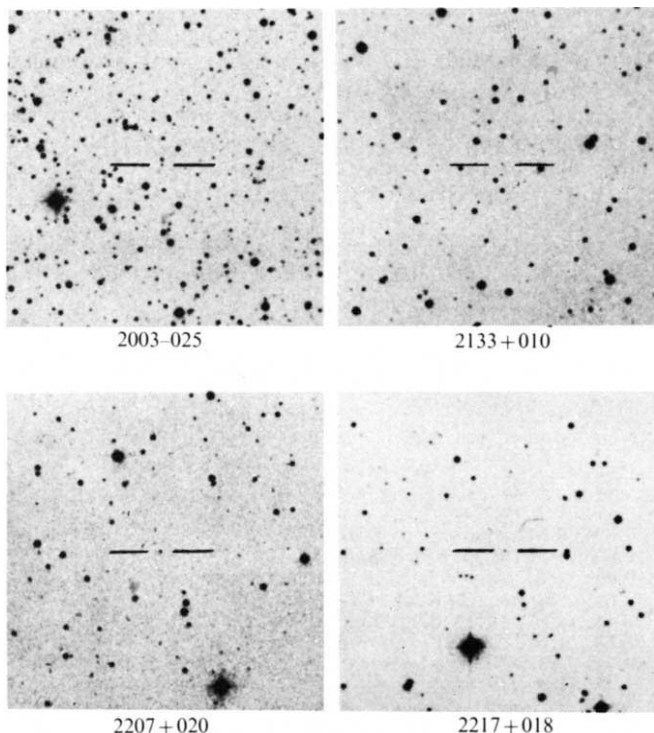


Fig. 1 Finding charts (approximate scale 10 arc s mm^{-1}) reproduced from the red prints of the Palomar Sky Survey.

spectrum QSOs of high redshift may be that few QSO candidates fainter than 19.5 mag have been studied spectroscopically.

The Parkes 2,700 MHz survey of the $\pm 4^\circ$ equatorial strip contains 341 sources above the completeness level of 0.35 Jy ($1 \text{ Jy} = 10^{-26} \text{ W Hz}^{-1} \text{ m}^{-2}$). We have used accurate radio positions measured at the Royal Radar Establishment, Malvern, to check the original Parkes identifications, and in some cases to propose new or revised identifications. One hundred of the sources can be identified confidently with stellar objects down to the limit of the Palomar Sky Survey prints. The radio structures of these QSO candidates have been studied at 962 and 1,666 MHz with the Mark II-III radio link interferometer at Jodrell Bank. Full details of these observations will be published elsewhere. Four steep spectrum sources were found to be essentially unresolved, and all have optical properties, which, from the discussion above, would be expected of high redshift QSOs.

The positions of the four QSO candidates are given in Table 1, and finding charts are given in Fig. 1. The properties of the objects are summarised in Table 2. They all have spectral indices equal to or steeper than -0.7 , and none shows evidence of a low frequency cutoff. This suggests that the sources do not possess intrinsically compact structures. Three are completely unresolved in our interferometric observations and thus have largest angular sizes of less than 0.5 arc s . The fourth ($2207 + 020$) may possess extended structure in addition to a compact component giving rise to 70% of the total intensity. Visual

magnitudes for the sources have been estimated from the Palomar Sky Survey prints. All are 19.5 mag or fainter. Colour estimates have been provided by J. G. Bolton for almost all the QSO candidates in our sample. Three of the four sources discussed here are amongst the small number (only seven) found to have no ultraviolet excess. Another of the seven, which has a flat radio spectrum, has a redshift of 2.66 (ref. 7).

Table 2 Radio and optical properties of compact QSO candidates

Source	Radio spectral index	Largest angular size (arc s)	Visual magnitude	Ultraviolet excess
2003 - 025	-0.7	<0.5	19.5	No
2133 + 010	-0.7	<0.5	20.0	Yes?
2207 + 020	-0.8	(~ 1)	19.5	No
2217 + 018	-0.7	<0.5	19.6	No?

Individually, the properties of small angular size or optical faintness cannot be taken as strong evidence that a QSO has an unusually high redshift. When, however, these properties are combined with a steep radio spectrum and lack of ultraviolet excess, we consider that such an object may well have a high redshift. Optical spectroscopy on 2003 - 025 and 2207 + 020 has recently been reported by Strittmatter *et al.*⁸. They confirm that both objects have ultraviolet deficits, but are unable to assign redshifts. Despite this, we suggest that all four objects are worthy of further spectroscopic investigation.

M.B. and D.S. acknowledge financial support from the Science Research Council.

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Received August 30, 1974.

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Table 1 Radio positions of compact QSO candidates

Source	Radio position (1950.0)			Position accuracy (r.m.s.) (arc s)
	RA (h min s)	dec. ($^\circ$ ' ")		
2003 - 025	20 03 32.22	-02 32 15.2		0.8
2133 + 010	21 33 19.56	01 04 48.1		1.3
2207 + 020	22 07 00.20	02 03 55.9		1.5
2217 + 018	22 17 58.05	01 49 46.6		1.4

Change of pulse characteristics of the Vela pulsar with frequency

BELOW 2,000 MHz the average pulse profile from the Vela pulsar (PSR 0833-45) shows a single peak with a sharp rise and a more gradual decay¹. At higher frequencies a broader and more complex pulse is observed, evidently comprising two distinct components^{2,3}. Observations at 1,400 and 4,830 MHz made with the Parkes 64-m telescope in November 1972 to clarify this frequency dependence can also be related to some γ -ray observations made by Albats *et al.*⁴ at the same time.

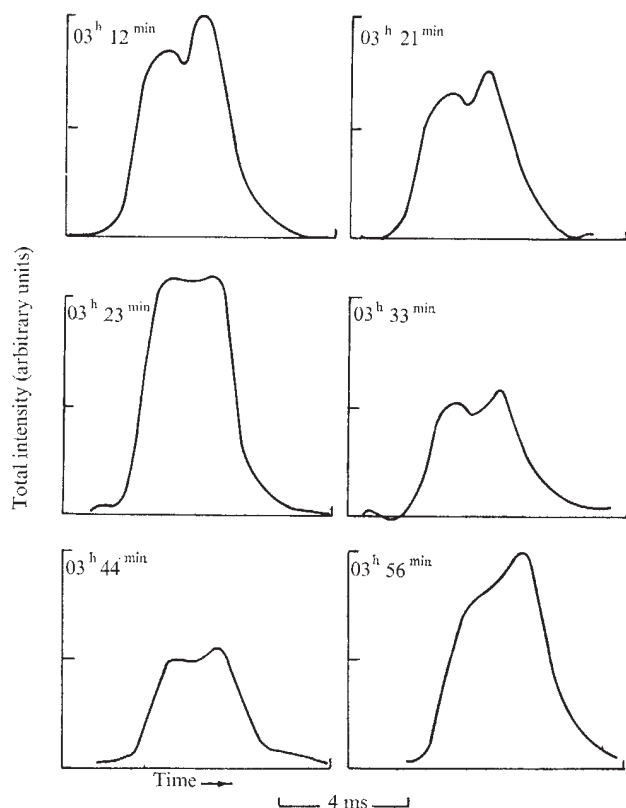


Fig. 1 Average 'total intensity' profiles measured at 4,830 MHz. Each profile is the sum of two integrations at orthogonal position angles of the feed. At each position angle 500 pulses were superposed. The times are Eastern Australian Standard Time on November 8, 1972.

Details of the radio observations are given in Table 1. A single receiver and linearly polarised feed were used at each frequency.

Figure 1 shows some examples of the 'total intensity' pulses measured at 4,830 MHz. The double structure is apparent, as is the time variability (over periods of a few minutes). Some of the variability must be intrinsic to the pulsar, since the relative amplitude of the two peaks also varies. Before the polarisation analysis was carried out the total intensity profiles were normalised.

Ideally, to determine which 4,830 MHz peak most nearly coincides with the 1,400 MHz pulse, simultaneous observations should have been made. But since the two receivers cannot be operated simultaneously the observations were made consecutively and connected by accurate timing measurements.

Table 1 1972 November observations of PSR0833-45*

Date	Frequency (MHz)	Bandwidth (MHz)	System temperature (K)
November 8, 1972	4,830	15	90
November 15-19, 1972 (inclusive)	1,400	0.1	120

* Average pulse profiles were measured by superposing many pulses, using a time base operating at the Doppler-shifted pulsar frequency and a sampling rate of 1/400 period (0.223 ms). Such pulse profiles were formed at a number of different feed position angles and the linear polarisation parameters of the average pulse were computed. The measurements were made in pairs, one feed position angle being followed by the position angle orthogonal to it. The sums of such pairs of integrations yield the total intensity pulse.

Several times on each observing day the pulse arrival time was measured against a timing pulse from a caesium-beam clock. Each measurement was accurate to about one-half of a sampling interval, or about 0.1 ms. From the measured arrival times at 1,400 and 4,830 MHz, and from the pulsar's known position⁵ and dispersion measure⁶, the barycentric period P_0 and its time derivative $\dot{P}$ were estimated. For JD 2441638.5 these were $P_0 = 0.08922348324 \pm 7$ s and $\dot{P} = 1.2508 \pm 0.002 \times 10^{-13}$. This value of P_0 , together with the value⁷ on JD 2441199.2 and one measured at Parkes on JD 2441934.5 (made available by V. Radhakrishnan and J. Brooks) yields values of $\dot{P}$ averaged over several months before and after our observations. These are 1.2477×10^{-13} and 1.245×10^{-13} , both very slightly smaller than our estimate.

The 'arrival times' used in the estimates relate to the half-intensity points on the leading edge of the pulse. To test the

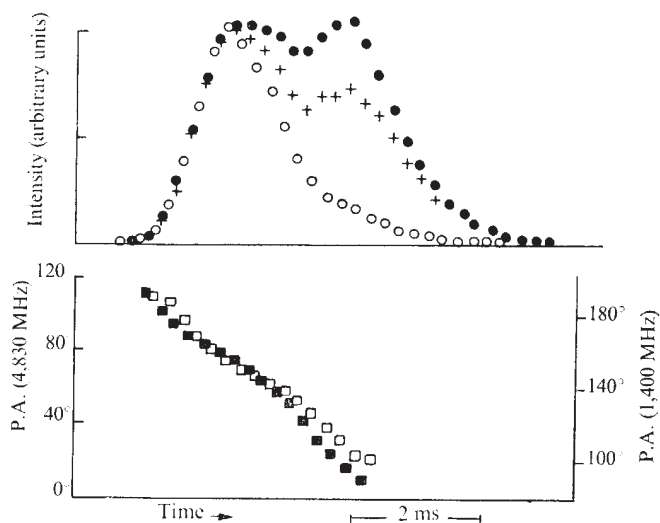


Fig. 2 Average pulse characteristics at 1,400 MHz (open symbols) and at 4,830 MHz (filled symbols). The crosses represent linearly polarised intensity at 4,830 MHz.

possibility that the second 4,830 MHz component more nearly coincides with the 1,400 MHz pulse, we added 1.8 ms—corresponding to the delay of the second component relative to the first—to the 4,830 MHz arrival time and repeated the calculation. There was no significant change in P_0 , but $\dot{P}$ increased to 1.256×10^{-13} , thus widening the disagreement with the long-term averages. We therefore associate the first component at 4,830 MHz with the single 1,400 MHz pulse.

Figure 2 shows the average profile at 4,830 MHz with that at 1,400 MHz superimposed. The leading edges have been accurately aligned. The shapes of the leading edges and the form of the polarisation position angle 'sweep' at the two frequencies agree well. The position angle at the first 4,830 MHz peak also agrees to within a few degrees with the value extrapolated from lower frequencies. Thus the 4,830 MHz pulse may be represented as the sum of two components (Fig. 3), the first being similar to the 1,400 MHz pulse. The energies E_1 and E_2 of the two components are each equal to about $20 \times 10^{-30} \text{ J m}^{-2} \text{ Hz}^{-1}$.

Pulse variability makes it extremely difficult to estimate a meaningful absolute spectral index for frequencies above 4,830 MHz. Writing $E_1 \propto f^{-\alpha}$ we find values of 2.2 or 4.8 for α , depending on which measurements^{3,5} near 8,000 MHz we combine with the present data. The relative spectral index is more easily estimated. The present results, together with those of Downs *et al.*³, indicate that E_2/E_1 varies approximately as

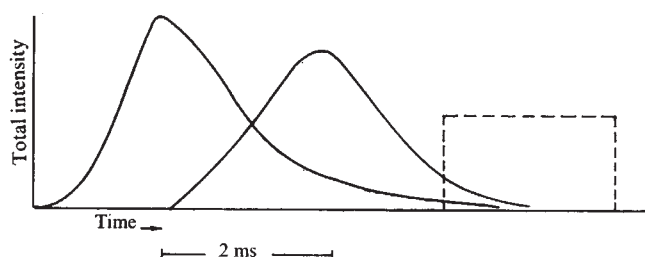


Fig. 3 The two pulse components at 4,830 MHz. The rectangle represents the arrival time of a possible γ -ray pulse as reported by Albats *et al.*⁴ The delay of the radio pulse due to dispersion has been removed.

$f^{2.3}$. The very much flatter spectrum of the second component suggests that a different emission mechanism is involved, but it is of interest that the position angle sweep through the whole pulse shows little frequency dependence.

Any pulses at frequencies beyond the radio domain might be expected to occur at the time of the second component. On November 16, 1972, Albats *et al.*⁴ attempted to detect pulsed γ rays in the 10–30 MeV range from the Vela pulsar. Figure 3 shows the temporal relation between the radio pulse and a possible γ -ray pulse that they report. It seems to lag behind both components of the radio pulse. The estimated uncertainty in our absolute timing measurement does not exceed 1 ms, and Albats *et al.* quote a similar uncertainty for their data. The time difference exceeds the sum of these uncertainties, but it is of interest that the γ -ray pulse more nearly coincides with the 'flatter spectrum' radio component.

We thank M. E. Ash, I. I. Shapiro and W. B. Smith of MIT Lincoln Laboratory for the use of their ephemeris of the Earth.

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New extragalactic variable sources from an 8-GHz survey

STUDIES of variable extragalactic radio sources have concentrated on sources with 'centimetre-excess' spectra, so that few sources without such spectra have been extensively monitored. We have used the NRAO 300-foot transit telescope at 2.7 GHz to make repeated, accurate observations of 48 sources selected by the Michigan 8-GHz survey¹ without further bias towards any radio spectral type. Variability has been detected in two radio galaxies without centimetre excesses and every quasistellar source in the survey has now been observed to vary at some frequency.

We observed every 8-GHz survey source less than 1' in diameter that would not be significantly confused using the 4.7' resolution of the 300-foot telescope at 2.7 GHz. A three-feed system was used to determine declination pointing corrections at every source transit, so that flux densities could be measured with standard errors of ~ 0.03 Jy from a single transit. The normalisation of the flux density scale among the seven observing periods between September 1972 and June 1974 was established to within 0.5% by observing ~ 200 nonvariable sources. Details of the observing and calibration procedures will be given by Kesteven and Bridle (in preparation).

Flux densities derived from different transits of a source within each of the seven observing periods have been averaged, as the flux density scatter between transits separated by only a few days is generally consistent with the errors computed for single transits. A χ^2 test was used to assess whether the scatter of the flux densities from different observing sessions is larger than expected from the errors in the individual measurements. We consider a source to be variable if the χ^2 probability that the observed scatter arises by chance is less than 0.1%, and possibly variable if it is between 0.1 and 1%. If some sessional flux densities were derived from only one transit the test was repeated deleting each such single observation in turn, and our assessment of source variability is based on the most mutually consistent data set. This procedure ensures that no source is considered variable on the evidence of a single discrepant transit which might have been affected by unknown instrumental effects.

The results for the 48 sources observed are summarised in Table 1. The flux densities are adjusted to the scale of Kellermann *et al.*²; the errors given for nonvariable sources do not include the uncertainty in normalising to this flux density scale as this does not affect our assessment of variability between the seven observing periods.

Twenty-one of the sources are variable at the 99.9% confidence level and three more at the 99.0% level; seventeen of these 24 are previously known variable sources. The measured flux densities of the five new variable and two new possibly variable sources are given in Table 2. Eleven previously-known variable sources did not vary significantly; the variable component(s) of these sources may make only a small contribution to the total flux density at 2.7 GHz or they may have been quiescent throughout the observing period (see Fig. 3 of Medd *et al.*⁴ for examples of such quiescence in variable sources).

The observations confirm the finding^{1,2} that the stellar objects in the 8-GHz survey tend to be variable on time scales of a few years whereas the radio galaxies do not. Nine of the fifteen confirmed QSS are variable at 2.7 GHz with variability indices from 0.030 to 0.243, and the remaining six have all varied at one or more other frequencies (see Table 1). Thus all of the confirmed QSS in the 8-GHz survey are now known to be variable. We have also found 8 of the 15 stellar objects without measured red shifts to be variable at 2.7 GHz

Table 1 Sources observed in this study (2.7 GHz)

Source name ¹	Other name	Optical identification ¹	No. of observations	Variable at 2.7 GHz?	Flux density range (Jy)*	$V_{2.7}$ †	Other refs for variability
0034-01	3C15	GAL	7	Yes	2.34 / 2.63†	0.058	
0035-02	3C17	GAL	7	No	3.94±0.04	<0.021	
0056-00	PK0056-00	QSS	7	No	1.93±0.03	<0.023	1
0106+01	PK0106+01	QSS	7	Yes	2.54 / 3.75	0.192	1, 2, 4, 8
0111+02	OC019	GAL	6	No	0.62±0.02	<0.058	
0112-01	P0112-017	BSO	6	No	1.11±0.04	<0.058	1, 2
0119+04	OC033	BSO	5	Yes	1.65 / 1.91†	0.073	
0122-00	PK0122-00	QSS	7	Possibly	1.04 / 1.21	0.076	1
0237-02	PO237-027	BSO	7	No	0.35±0.05	<0.200	
0240-00	3C71	GAL	7	Yes	2.80 / 3.23†	0.071	
0305+03	3C78	GAL	7	No	4.82±0.02	<0.016	
0336-01	CTA26	QSS	7	Yes	1.98 / 3.25	0.243	1, 2, 3, 4, 5, 6, 8
0420-01	PK0420-01	QSS	7	No	1.23±0.04	<0.058	1, 2, 4
0422+00	PK0422+00	?	7	Yes	0.49 / 0.92	0.305	1, 2
0440-00	NRAO190	BSO	7	Yes	2.47 / 2.92	0.083	1, 2, 3, 4, 8
0500+01	OG003	□	7	Possibly	2.14 / 2.35†	0.047	
0723-00	DW0723-00	NSO	7	Yes	1.94 / 2.25	0.074	1, 2, 4, 7
0736+01	PK0736+01	QSS	6	No	1.97±0.12	<0.080	1, 4, 5
0743-00	OI-072	NSO	7	No	1.37±0.04	<0.051	1, 4
0829+04		NSO	7	No	0.61±0.05	<0.102	1
0906+01	PK0906+01	QSS	7	No	0.86±0.03	<0.059	1, 2, 7
0922+00	OK037	QSS	7	Yes	0.70 / 0.82	0.079	1
1055+01	PK1055+01	BSO	7	Yes	2.75 / 3.07	0.055	1, 2, 3, 4, 5, 6
1148-00	PK1148-00	QSS	7	Possibly	2.35 / 2.56†	0.043	
1219+04	PK1219+04	BSO	6	Yes	0.59 / 0.79	0.145	1, 2
1226+02	3C273	QSS	6	Yes	39.2 / 44.6	0.064	1, 2, 3, 4, 5, 6
1330+02	3C287.1	GAL	7	No	1.70±0.03	<0.029	
1434+03	PK1434+03	□	7	No	1.83±0.01	<0.014	
1456+04	4C4.49	?	6	No	0.74±0.03	<0.060	
1532+01	PK1532+01	BSO?	7	Yes	1.07 / 1.18	0.049	1
1535+00		?	7	Yes	0.84 / 1.06†	0.116	
1546+02	OR078	QSS	7	Yes	1.04 / 1.26	0.096	1, 2, 7
1555+00	DW1555+00	BSO	7	No	1.36±0.03	<0.030	1, 2, 4, 5
1635-03		GAL	6	No	0.48±0.02	<0.083	
1648+01		□	7	Yes	0.83 / 0.96	0.073	1
1741-03	P1741-038	OBSC	7	Yes	1.90 / 2.65	0.165	1, 5
1949+02	3C403	GAL	7	No	3.35±0.03	<0.021	
2044-02	3C422	GAL?	7	No	1.43±0.04	<0.042	
2059+03	OW098	QSS	7	Yes	0.61 / 0.72	0.083	1
2128+04	PK2127+04	□	7	No	2.94±0.02	<0.012	
2131-02	OX-053	BSO?	7	Yes	1.58 / 2.39	0.204	1, 2
2134+00	P2134+004	QSS	7	Yes	7.03 / 7.47	0.030	4
2210+01	PK2210+01	□	7	No	1.67±0.01	<0.015	
2216-03	PK2216-03	QSS	7	No	1.23±0.02	<0.025	1, 2, 4
2254+02	OY091.3	QSS	6	No	0.35±0.01	<0.056	1
2318+04	OZ031	BSO	6	Yes	1.07 / 1.32†	0.105	
2332-01	OZ-055	BSO	7	No	0.54±0.03	<0.101	1
2335-02	OZ-060	BSO	7	No	0.64±0.04	<0.071	

*The mean flux density is given for sources that did not vary significantly at the 99% confidence level.

†See Table 2 for detailed flux density tabulation.

‡The 'variability index', $V_{2.7} = (S_{\max} - S_{\min}) / (S_{\max} + S_{\min})$, is a normalised measure of the amplitude of the observed variations.

with variability indices from 0.049 to 0.204, a range very similar to that exhibited by the confirmed QSS. Five further stellar objects in the survey are known to have varied at some other frequency. The only 8-GHz stellar objects not yet observed to vary are 0237-02 and 2335-02.

These results are consistent with those of other studies of radio-source variability (such as ref. 9) as all of the stellar objects in the 8-GHz survey have centimetre-excess spectra. The very high incidence of variability among stellar objects with such spectra (93%) suggests that nonvariable objects of this type are rare, if indeed they exist at all.

Our results for radio galaxies are more surprising. Of the ten radio galaxies observed, two (0111+02 and 1635-03) have centimetre-excess spectra and neither has

yet been found to vary at any frequency. Unexpectedly in view of most earlier studies, two of the remaining eight radio galaxies have shown significant variations at 2.7 GHz. These are 0034-01 (3C15) and 0240-00 (3C71). Hazard *et al.*¹⁰ found 0034-01 at 410 MHz to have three main components each ~2" in diameter, the outer components being ~10" apart. The innermost component coincides with the elliptical galaxy that is the identification. 0240-00 has 25% of its 2.7-GHz flux density in a component <1.5" in diameter¹¹ located in the nucleus of the Seyfert galaxy NGC-1068. It has previously been suspected of being variable at millimetre wavelengths¹². In both 0034-01 and 0240-00 at least part of the radio source lies within the associated galaxy, but the variable components do not dominate the

Table 2 Flux densities of new variable sources at 2.7 GHz

Source	Date	Flux density (Jy)	Date	Flux density (Jy)
<i>a</i> Variability significant at 99.9% level				
0034-01	September 72	2.50 $\pm$ 0.04	November 73	2.34 $\pm$ 0.03
	January 73	2.50 $\pm$ 0.10	February 74	2.63 $\pm$ 0.04
	April 73	2.47 $\pm$ 0.04	June 74	2.42 $\pm$ 0.02
	August 73	2.50 $\pm$ 0.02		
0119+04	September 72	1.79 $\pm$ 0.04	February 74	1.79 $\pm$ 0.02
	August 73	1.91 $\pm$ 0.02	June 74	1.65 $\pm$ 0.02
	November 73	1.78 $\pm$ 0.02		
0240-00	September 72	3.08 $\pm$ 0.03	November 73	3.23 $\pm$ 0.03
	January 73	2.85 $\pm$ 0.04	February 74	3.16 $\pm$ 0.03
	April 73	2.80 $\pm$ 0.04	June 74	3.04 $\pm$ 0.03
	August 73	3.08 $\pm$ 0.04		
1535+00	September 72	1.06 $\pm$ 0.02	November 73	0.97 $\pm$ 0.02
	January 73	0.87 $\pm$ 0.01	February 74	0.92 $\pm$ 0.02
	April 73	0.93 $\pm$ 0.02	June 74	0.91 $\pm$ 0.01
	August 73	0.84 $\pm$ 0.01		
2318+04	September 72	1.28 $\pm$ 0.02	November 73	1.20 $\pm$ 0.04
	January 73	1.28 $\pm$ 0.03	February 74	1.11 $\pm$ 0.03
	August 73	1.32 $\pm$ 0.03	June 74	1.07 $\pm$ 0.02
<i>b</i> Variability significant at 99.0% level				
0500+01	September 72	2.35 $\pm$ 0.03	November 73	2.33 $\pm$ 0.03
	January 73	2.28 $\pm$ 0.07	February 74	2.32 $\pm$ 0.03
	April 73	2.14 $\pm$ 0.03	June 74	2.34 $\pm$ 0.02
	August 73	2.23 $\pm$ 0.02		
1148-00	September 72	2.53 $\pm$ 0.06	November 73	2.56 $\pm$ 0.03
	January 73	2.36 $\pm$ 0.04	February 74	2.54 $\pm$ 0.03
	April 73	2.51 $\pm$ 0.05	June 74	2.35 $\pm$ 0.03
	August 73	2.47 $\pm$ 0.03		

high frequency spectrum as in the well known variable radio galaxies such as 3C84 (NGC1275). The variations observed in 0034-01 and 0240-00 are of correspondingly small amplitude, with variability indices of 0.058 and 0.071 respectively. It will be important to determine whether there are variable components in other multi-component radio galaxies like 0034-01, and to establish the location of such variable components within the radio structures.

Five of the six unidentified sources with centimetre excesses are also variable at 2.7 GHz, with variability indices that range from 0.047 to 0.305. The variability of these sources suggests that they are likely to be quasistellar objects.

We thank the Director of the National Radio Astronomy Observatory for providing observing time and the National Research Council of Canada for support. The NRAO is operated by Associated Universities Inc. under contract with the National Science Foundation.

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Received July 22, 1974.

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Infrared emission in R CrB type stars

INFRARED emission has been observed in R CrB type stars and is considered to be the reradiation of the absorbed visual light by graphite grains^{1,2}.

The lack of correlation between change of star brightness and infrared emission^{3,4} has been explained in terms of condensation of grains in discrete clouds escaping from the star in random directions. Under this condition we could observe infrared emission without any corresponding change in star brightness. In April and May 1972, however, the observed brightness of one R CrB star decreased about 6 mag, but no changes of infrared flux were observed.

We consider here a possible interpretation of this effect. It seems possible that in the escaped shell nuclei of grains might form in the shape of Platt particles. Such graphite particles with demensions from 3 to 30 Å will be like non-saturated molecules, the more so as it is likely that a graphite structure with vacancies will grow up. In these

particles absorption is a quantum-mechanical process resulting from electron jumps between different Fermi levels. For quasi-metallic particles the longest wavelength will be absorbed by one electron, when it jumps from the highest filled to the lowest empty Fermi level. Its wavelength is given approximately by $\lambda \approx 400L$, where L is the dimension of the particle⁵. Platt particles will re-emit photons isotropically which were absorbed from an incident beam with very small shifting to the red. These particles cause the extinction a few hundred times greater than classical grains with the same mass; however, they will not reradiate in the infrared region.

The growth of Platt particles to a size of order 10^{-7} cm (by accretion C , C_2 and so on, and perhaps coalescence) leads to the formation of the classical nuclei of grains. Further growth will form graphite grains with the radii between 2×10^{-6} and 2×10^{-5} cm whose re-emission in the infrared completely fits the observational data for R CrB and SY Sgr stars⁶.

It is possible that infrared emission is shifted in time relative to the light minimum by an amount appropriate to the necessary period of formation of the classical graphite grains. This time shift depends on the initial conditions of the escaped shell, particularly on the density and velocity of escape. For that reason this time shift may be different for particular light minima.

This interpretation is confirmed by the fact that light polarisation of R CrB star observed by Coyne and Shawl⁷ is strictly correlated with the light curve. At light minimum, and some time after, its light polarisation increases with the decrease of the wavelength which agrees with the behaviour expected for polarisation caused by Platt particles⁸. For the period before minimum, polarisation is considerably lower and its dependence on the wavelength indicates an origin from grains with relatively large sizes.

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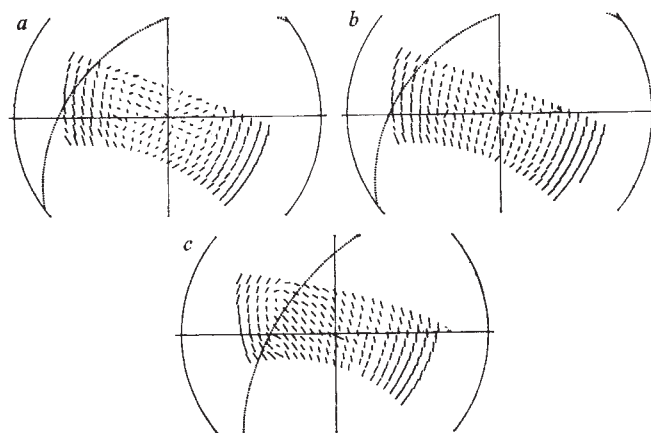


Fig. 1 Linear polarisation of the night sky at 5,300 Å. The diagrams are centred at the ASP. The north ecliptic pole is at the top. Vertical line, antisolar meridian; horizontal line, the ecliptic plane; dotted line, galactic equator. Part of the circle representing 90° solar elongation is shown. Each measurement is represented by a line with a length proportional to $\log(1 + pI)$, where pI is polarised intensity in S_{10} (V) units. a, August 25, 1973; b, August 26, 1973; c, July 29, 1973.

Full data will be published elsewhere; here, we discuss 63 nights of observation from May 1973–June 1974.

Our observations consist of regular measurements of the Stokes parameters, I , Q , and U , of the night sky radiation at 5,300 Å (half-intensity filter width 53 Å) at fixed altitudes on the N–S meridian. Meridian observations are preferable because they allow measurement of each region at its maximum altitude when the influence of tropospheric scattering is minimal. Initially, we observed only on the 180° meridian at altitudes of 35°–75° at 5° intervals, using a 5° diameter field of view and an integration time of $\tau = 2$ min to ensure a high precision in the measured position angle, χ , of the polarisation plane. For more complete coverage of the night sky, we now observed on both the north and south meridian, at altitudes of 35.0°, 41.9° to 83.3°, and 90°, again using a 5° diameter field of view but with an integration time of only 30 s. A reduction in τ was necessary in order to maintain the same spatial resolution—about 5 square degrees. For $\tau = 30$ s, the typical uncertainty in χ is 6°—small enough to allow the detection and monitoring of significant detail in the polarisation. The possible influence of tropospheric scattering is minimised by observing above an altitude of 35° (and on the N–S meridian). We also monitored tropospheric effects by observing a fixed set of points, all with a declination of 20.7°, at various altitudes. We found that at Haleakala, tropospheric scattering at 5,300 Å is usually unimportant above an altitude of 35°. Occasionally though—for instance, during enhanced airglow activity—close examination of the data for tropospheric scattering effects may be necessary.

Polarisation maps can be prepared from the data; these are plots of the polarised intensity vector in an ASP-centred diagram (Fig. 1). Maps of Q , U and pI ($= [Q^2 + U^2]^{1/2}$) can also be obtained¹.

The polarisation maps show several effects: first, sufficiently far away from the ASP (and from the galactic plane) the polarisation plane tends to be normal to the scattering plane defined by the Sun, the Earth, and the zodiacal dust particle. This orientation can be expected if the dust particles are spherical or non-spherical and randomly oriented.

Second, within about 30° from the ASP, there are significant deviations from this orientation. They take the form of two polarisation patterns: pattern *a*, with relatively small values of pI (typically less than 0.8 S_{10} (V) near the ASP), and with nearly random orientation of the polarisation plane (Fig. 1*a*); pattern *b*

Polarisation in the night sky at 5,300 Å

We have presented observations¹ of the linearly polarised intensity, pI , at 5,080 Å (that is, polarisation, $p \times$ intensity, I), of radiation in the night sky. Contour plots of pI showed a displacement of the minimum of pI away from the antisolar point (ASP). A significant angular structure, and day-to-day variations were also evident. Here, we report observations of pI and of the orientation angle, χ , of the polarisation plane of radiation at 5,300 Å in the night sky. Measurements have been taken since May 1973 with a photoelectric polarimeter at the University of Hawaii (J. L. Weinberg, unpublished). Several modifications have been made recently, the most important being the analog–digital conversion of data recording. Digitisation allows faster data reduction and improves measurement precision, which is now limited by instrumental polarisation and photon statistics.

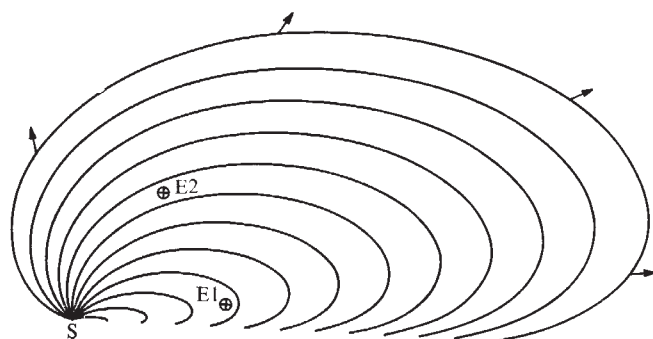


Fig. 2 Schematic representation of an expanding shock front from the solar wind. The Sun is at S, the Earth at two alternative positions, E1 and E2 (see text).

has pI values typically larger than $1.5 S_{10}$ (V) near the ASP, with the polarisation plane tending to be normal to the ecliptic plane (Fig. 1b).

Third, a pattern usually persists for as long as a week, which was our typical useful observational period during a lunation, with only minor changes occurring in that pattern. At least one example of a sudden change was, however, observed, between August 25 and 26, 1973 (compare Fig. 1a and b).

Fourth, the influence of the Milky Way on the polarisation is seen clearly when the galactic equator crosses the ecliptic within about 30° of the ASP. There is a tendency for the polarisation vectors to be rotated normal to the galactic equator (Fig. 1c).

Fifth, the polarised intensity does not vanish at the ASP; that has already been established¹.

The observed effects can be understood if the zodiacal dust is non-spherical and if, at distances from the Sun typically greater than 1 AU, it can have preferred directions of orientation, that is, be partially aligned. Observational evidence of the existence of appreciably non-spherical grains has been presented elsewhere (refs 1-3, and R. D. Wolstencroft, and J. C. Brandt, unpublished). The fact that at elongation angles, ϵ , of less than 90° the polarisation plane tends to be normal to the scattering plane implies that close to the Sun the particles are more nearly oriented randomly. This conclusion is supported by the observations of Sparrow and Ney⁴, who detected no change in the zodiacal light at 90° elongation over a 4-yr period.

We suggest that the polarisation patterns, *a* and *b* (Fig. 1a and b) correspond to two states of particle orientation: an undisturbed state (Fig. 1b) in which the particles are partially aligned; and a disturbed state (Fig. 1a) corresponding to random orientation. We have suggested (ref. 1, and R. D. Wolstencroft and J. C. Brandt, unpublished) that the interaction of the dust with the solar wind plasma and with the interplanetary magnetic field, changes the orientation and the degree of alignment of the grains. Details of the theory of the interaction need to be established, particularly the way in which significant changes in grain orientation are produced sometimes in only one week, or less. Nevertheless, we can suggest models which relate the observed changes of the polarisation pattern to disturbances in the solar wind plasma or of the magnetic field.

Consider the influence of the disturbed solar wind. As the shock front associated with a solar wind disturbance moves outward from the Sun and into the domain of grains which are usually aligned, an expanding region of disoriented grains forms in that domain (that is, at distances typically greater than 1 AU). Consequently, the polarisation pattern in the antisolar hemisphere changes from *b* to *a* (Fig. 1a and b). The effect of the zone of disoriented grains on the polarisation pattern depends on the position of the Earth. If the position is that of E1 in Fig. 2, evidently only part of the antisolar hemisphere shows a disturbed pattern. If the position is E2, the entire hemisphere is affected. But the angular size of the disturbed pattern also depends on the scattering function of the grains and may be

smaller than the size of the region in which the orientation of the grains has been disturbed. When the line of sight through the zone of disoriented grains becomes long enough, observable changes in the polarisation pattern will occur; it will occur a few days after the shock front has encountered the Earth. The change in pattern will be gradual and should be most noticeable close to the ASP where the particles beyond the front contribute relatively little to the polarised intensity.

Now consider the influence of regions of disordered magnetic field, which are associated with sector boundaries. As in the case of shock fronts from the solar wind we assume that the passage of the sector boundary disorients the grains. If the time needed for the particles to be re-aligned were greater than the time that occurred between boundary passages (typically one week) then the grains would be permanently disordered. The orientation of the polarisation within 30° of the ASP given by pattern B is inconsistent with permanent disorientation of the grains. When the sector boundary reaches the Earth we should see a sharp edge dividing the polarisation pattern into ordered and disordered zones (corresponding to patterns *b* and *a*, respectively). As the boundary moves past the Earth, this edge moves towards the ASP and into the morning hemisphere. For reasons related to the scattering function, already mentioned, the moving edge may not be well defined, however, and may therefore not be detected until it has come close to the ASP.

As the observed changes in polarisation patterns depend on the position of the Earth relative to the disturbances in the interplanetary medium, as well as on other factors (such as the scattering function of the dust), correlating pattern changes and events in the interplanetary medium is not a trivial task. In fact, we have not been able to attribute the sudden change between August 25 and 26, 1973, to any interplanetary event. We are not discouraged, however; if a clear correlation between pattern changes and events in the interplanetary medium were established, there would exist a unique method of observing and monitoring the interplanetary medium in regions as yet inaccessible to space probes.

This work was supported financially in part by the National Science Foundation.

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Received May 14; revised July 15, 1974.

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Arithmetic of ice ages

THE Milankovitch hypothesis is that glaciations occur when the Northern Hemisphere receives relatively little summer heat from the Sun, because of astronomical factors that alter the orientation of the Earth's axis and the eccentricity of its orbit. A new time scale for the ocean-bed record of the past eight glaciations is provided^{1,2} by the magnetic reversal at the start of the Brunhes epoch, 700,000 yr ago. In addition, Vernekar's recalculated insolation tables³ are now available. For a popular account of climatic change⁴, I wanted to illustrate the opportunity thus provided, for a re-evaluation of the Milankovitch hypothesis.

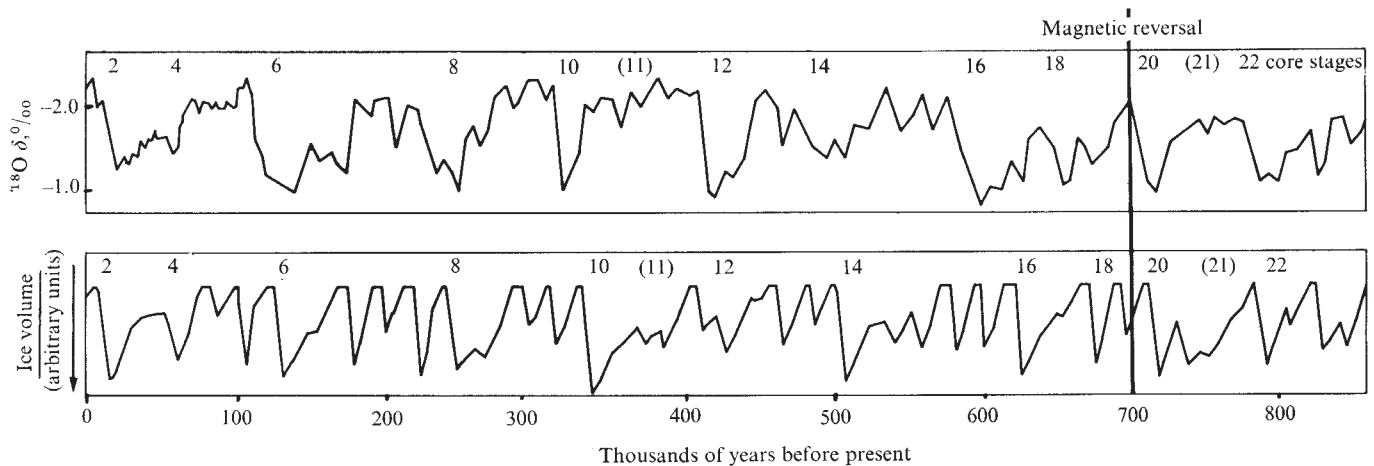


Fig. 1 Upper, measured oxygen-isotope changes in marine organisms from Fig. 9 of ref. 1; low points indicate a large volume of ice. They are plotted against depth in core and so the dating is uncertain except at the magnetic reversal 700,000 yr BP. Lower, ice volume derived by arithmetic as described in the text, from 50°N insolation data given in ref. 3 (inverted, arbitrary units).

An inspection of Vernekar's tables suggested that the crucial zone for summer sunshine was not at the Arctic Circle, as has often been supposed, but at 50°N. The theory of ice sheets growing from the bottom up^{5,6} and the discovery of the English Channel glacier^{7,8} fed from the seabed south of Ireland make this choice of latitude glaciologically plausible.

Suppose, now, that a decline in summer sunshine at 50°N below a certain level allows the volume of glaciers and ice sheets to grow in simple proportion to the deficit, while summer sun-

Table 1 Comparison of corrected ocean-core dates of maximum glaciation, taken from Fig. 4 of ref. 2, and the dates deduced arithmetically.

Core stage	Ocean-core date (to nearest 5,000 yr)	Arithmetical date (to nearest 1,000 yr)
2	20	16
4	65	62
6	110 or 140	133
8	245	248
10	330	344
12	420	429
14	505	509
16	595	626
18	665	676

Dates in thousands of years BP.

shine above that level melts ice with a different proportionality. For the point of equilibrium I take 17 langley per day (1 langley = 1 calorie cm⁻²) above the 1950 value of 847 langley per day. Glacial maxima or minima are then predicted at the dates when the summer insolation at 50°N crosses the +17 level.

Simple summing, by 1,000-yr steps, of the surpluses or deficits above or below +17 indicates in arbitrary units the extent of freezing or melting between the maxima and minima. With a melting rate of unity, a freezing rate of 0.22 gives a realistic curve for the most recent glaciation (the past 78,000 yr); melting the ice is easier than making it. The same proportionalities are then applied over the past 860,000 yr. Finally, as surplus sunshine cannot melt ice that is not present, a limiting value for the reduction in ice volume can be assumed. The running totals then predict the amount of ice in the world. Figure 1 compares the resulting arithmetical curve with measurements of the oxygen-isotope ratios in marine fossils¹, which are also thought to be proportional to the amount of ice in the world. Table 1 compares the dates of maximum glaciations

arrived at arithmetically, with the corrected dates deduced by Emiliani and Shackleton² who take account, as best they can, of variations in ocean-bed sedimentation rates.

Meteorological processes are so notoriously nonlinear that my assumptions are almost frivolous. The matches between the curves of Fig. 1 and the dates of Table 1 are very much better than they deserve to be unless the Milankovitch effect is indeed dominant. The arithmetical curve captures all the major variations and the core stages can be identified with little ambiguity. The defects include an excessive warm peak 170,000 yr ago, and excessive freezing in the more recent parts of core stages 11 and 21. On the other hand, the arithmetical curve registers two known events not conspicuous in Shackleton's oxygen-isotope curve, namely the cooling at 90,000 yr ago^{9,10} and the warming at 100,000 yr BP required by the speleothem data³.

Other processes are at work, including the 2,500-yr oscillation¹¹ that correlates with ¹⁴C production in the atmosphere, and hence with solar events rather than with the Milankovitch effect. Nevertheless the variations in summer sunshine available for melting the snow of the Northern Hemisphere plainly determine the first-order pattern of past glaciations. One can even put an order of magnitude to the process. A surplus of one langley per day above the +17 level, at 50°N in summer, will melt sufficient ice to change the oxygen-isotope ratio of ocean water at a rate of 0.01‰ per thousand years.

Extrapolation of the curve gives a first-order forecast (Fig. 2) for the 'next' ice age, which began 5,000 yr ago and will end 119,000 yr from now. Broecker and van Donk¹² arrived at a broadly similar forecast by more general reasoning from the insolation predictions. This ice age looks like a relatively slow starter. The theory, though, is of widespread snow that fails to

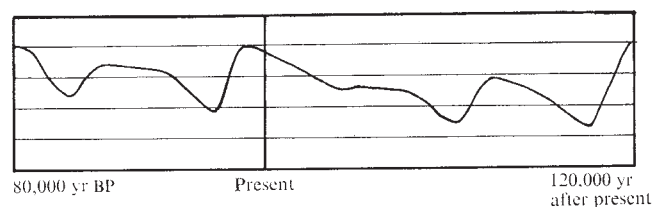


Fig. 2 Enlargement of the arithmetical curve for the most recent (Würm/Wisconsin) glaciation and an extrapolation for 120,000 yr into the future by the same arithmetical rules.

melt in the vicinity of 50°N in summer, so that large areas of North America, northern Europe and the USSR will have to be encrusted with ice sheets during the next few thousand years, to fulfil the expectations of Fig. 2.

I thank Dr N. J. Shackleton for suggestions and advice, and Professor H. H. Lamb for encouragement.

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Received July 22; revised September 5, 1974.

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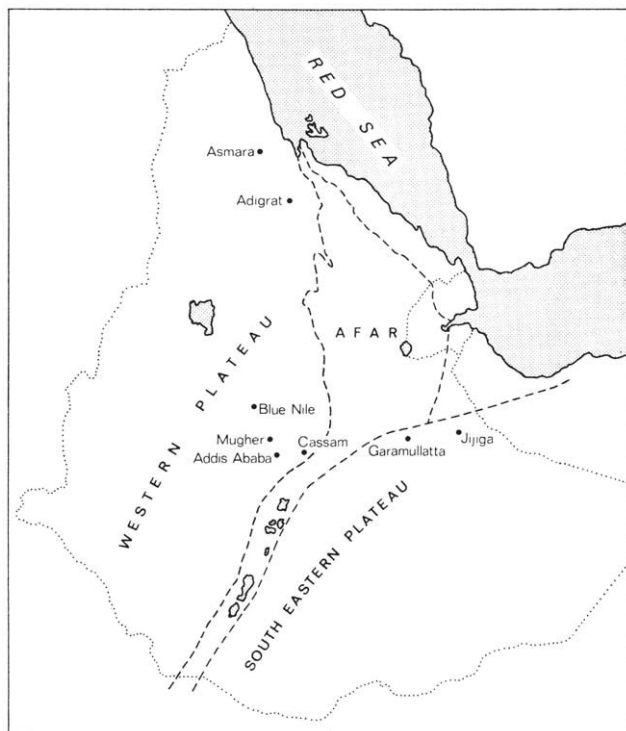


Fig. 1 Profile locality map of Ethiopia.

New dates from the Ethiopian plateau volcanics

INTERPRETATION of geological events in Ethiopia has been severely limited by the lack of isotopic age data from the plateau volcanics. Volcanism was thought to have begun in the Eocene—an idea based chiefly on ages from the Blue Nile Gorge basalts¹. These basalts have been resampled and ages of 23 to 27 Myr determined². Recent research involving the measuring and sampling of volcanic profiles on the Western and South Eastern Ethiopian Plateaus, has made it possible to sample the basal flows which immediately overlie Mesozoic sediments. We report here nine new K–Ar dates from the Mugher, Adigrat, Garamullatta, and Jijiga profiles (Fig. 1.) The data are presented in Table 1 together with brief petrographic descriptions. All

samples contain up to 12% intersertal glass; whole rock samples were analysed. The errors quoted are at the 60% confidence limit and based on replicate analyses.

The ages are striking both in their stratigraphic consistency and in the general similarity between profiles. Although it may be argued that they do not represent the oldest volcanics of the Ethiopian volcanic episode, nevertheless they do indicate that a considerable part of the plateau sequence is very much younger

Table 1 Sample data

Sample No.	Profile	Position in profile	%K	Vol. ⁴⁰ Ar rad. s.c.c. g ⁻¹ × 10 ⁻⁶	% ⁴⁰ Ar rad.	Age (Myr)	Petrography
30410	Jijiga	Second flow	0.93	0.8550	74.6	22.8 ± 0.7	Fine-grained sparsely feldspar-phyric. Plag. (An ₅₂), pale brown augite and abundant opaque ore. 10% clear green isotropic glass.
30409		Basal flow	0.72	0.6840	61.0	23.6 ± 0.7	Feldspar-phyric basalt. Plag. (An ₅₅₋₆₀ Zoned) in matrix of plag. (An ₅₃), pale brown augite and opaque ore. <5% clear glass.
30903	Adigrat	300 m from base	0.70	0.6461	78.4	22.8 ± 0.7	Feldspar basalt. Phenocrysts (approx. An ₅₅) in sub-trachytic texture of plag.laths, augite, and opaque ore. <1% glass.
30888		Second flow	0.61	0.5766	57.4	23.5 ± 0.7	Small clusters of slightly altered olivines in matrix as below. <5% green glass.
30884		Basal flow	0.62	0.7286	66.6	29.2 ± 0.7	Medium-fine grained aphyric basalt. Ophitic texture—plag.laths (An ₅₅) enclosed in pink titanite with abundant magnetite. Approx. 5% altered olivine 7–8% pale green glass.
30940	Garamullatta	200 m from base	0.72	0.4836	49.3	16.7 ± 0.7	Medium-fine grained feldspar basalts. Small plag. phenocrysts (An ₆₄) in sub-trachytic matrix composed of plagioclase, pale-brown augite, fresh olivine and opaque ore. Approx. 10% pale brown, isotropic glass.
30945		15 m from base	0.53	0.4824	59.8	22.7 ± 0.7	
30954	Mugher	Second flow	0.86	0.7137	65.1	20.6 ± 0.7	Porphyritic basalt. Clinopyroxene phenocrysts and clusters in very fine sub-trachytic matrix with abundant opaque ore. 10–12% green isotropic glass.
30948		Basal flow	1.07	0.8451	78.7	19.8 ± 0.7	Porphyritic basalt. Clusters of twinned, zoned plagioclases, pyroxenes and rare olivines in a fine matrix of similar composition. Less abundant opaque ore. Approx. 10% greenish-brown glass.

$$\lambda_e = 0.584 \times 10^{-10} \text{ yr}^{-1}; \lambda_\beta = 4.72 \times 10^{-10} \text{ yr}^{-1}; {}^{40}\text{K} = 1.19 \times 10^{-11} \text{ mol/mol K.}$$

than originally inferred. This is in good agreement with the new Blue Nile data, quoted above, and with an age of 20.9 Myr from the Cassam sequence³.

These K-Ar data cast considerable doubt on the theory that the volcanism began in the Eocene or early Oligocene⁴ since none of the ages so far obtained exceeds 30 Myr.

P. W. J. acknowledges support from the Natural Environment Research Council.

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Possible environmental index in tropical reef corals

PRELIMINARY results from a mineralogical study indicate that hermatypic corals may preserve in their skeletons a continuous record of noncarbonate sediments that were suspended in the waters in which they grew. Studies of coral structure, skeletal chemistry, and skeletal mineralogy¹⁻⁶ have tended to neglect the significance of this trapped material. Our findings suggest that any comparison between the abundance of trace elements in corals from different areas may be questioned, as may those studies which suggest that large amounts of silica and aluminium are incorporated in skeletal carbonate.

This study was restricted to noncarbonate detrital fractions in the skeletal cavities of live, tropical reef corals which were free of macroborings filled with sediment. Specimens were from the eastern coast of the United States, the Gulf of Panama, and the barrier reef off Belize. They were prepared by coarse crushing freshcut, weighed coral fractions; dissolution at room temperature and pH 11 in ethylenediaminetetraacetic acid (EDTA)⁷; treatment with 30% H₂O₂ to remove oxidisable organic material; drying and weighing of the residue; and then mounting the residue on glass slides for X-ray diffraction

analyses. Mineral components were identified using standard analytical techniques⁸. Annual radial density bands, recorded in X radiographs^{9,10}, were used to estimate growth increments in coral sections (Fig. 1).

Detailed microscopic examination of both freshly broken surfaces and surfaces etched with acid revealed some skeletal cavities which had been capped with a dissepiment after they had been partially filled with detrital material (Fig. 2). The dissepiments were commonly arched to accommodate trapped sediment, indicating that the polyps were alive when the sediment entered the skeletal cavities. As coral polyps can normally reject particulate material which is smaller than silt¹¹, the sediment probably entered the skeletal cavities when the polyp was damaged or destroyed, and became sealed into the skeleton after polyp regeneration or recovery of a segment of the coral

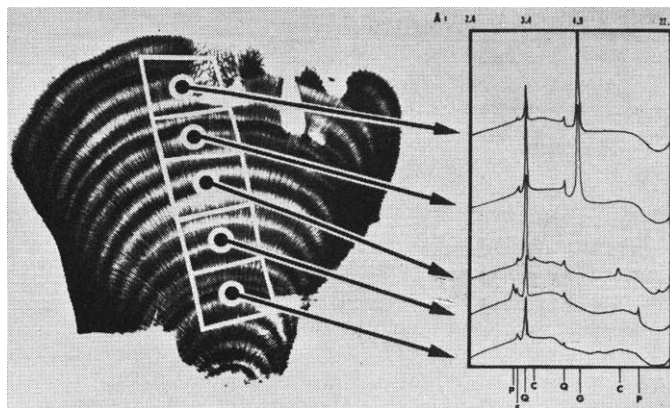


Fig. 1 X radiograph of *Pavona gigantea* showing variation in composition of noncarbonate detrital fraction relative to annual paired light and dark density bands. Note the appearance of gibbsite (4.85 Å) approximately six years ago. C, chlorite; T, talc; G, gibbsite; Q, quartz; F, feldspar.

colony. This type of material, however, was not found embedded in the skeletal carbonate matrix.

X-ray diffraction analysis of residue in specimens from the eastern United States showed the mineral suite: chlorite, gibbsite, illite, kaolinite, montmorillonite, quartz, talc, and several feldspars (Table 1). As considerable mineralogical variation occurs in detrital material from corals of the same species, the mineral suite is not species specific. It is, however related to the character of suspended or resuspended material

Table 1 Amount and composition of noncarbonate detrital material in corals

Coral sample and location	Approximate range of years in the past	Amount of entrapped sediment (p.p.m. by weight)	Mineral suite*
<i>Solenastrea hyades</i> (Dana)	present-92	580	I-T-KC-G-Q-F
North Carolina Shelf			
<i>Solenastrea hyades</i> (Dana)	present-91	103	M-I-T-KC-Q-F
North Carolina Shelf			
<i>Solenastrea hyades</i> (Dana)	present-117	150	I-T-KC-G-Q-F
North Carolina Shelf			
<i>Solenastrea hyades</i> (Dana)	present-50	750	I-G-Q-F
South Carolina Shelf			
<i>Solenastrea hyades</i> (Dana)	unknown	1,550	I-T-KC-G-Q-F
Florida Bay			
<i>Pavona gigantea</i> Verrill	1-16	171	T-G-Q-F
Gulf of Panama			
<i>Montastrea annularis</i>	2-16	243	I-G-Q
(Ellis and Solander) Belize			

*M, montmorillonite; I, illite; T, talc; KC, kaolinite-chlorite; G, gibbsite; Q, quartz; F, feldspars and plagioclases.

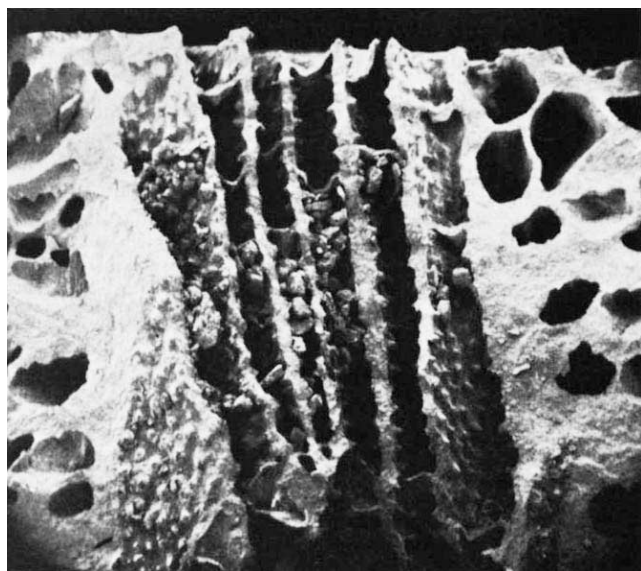


Fig. 2 Detrital material trapped in corallite of *Solenastrea hyades* (Dana) from North Carolina ($\times 16$). Note dissepiment cappings.

in the surrounding water masses¹²⁻¹⁴. No evident chronological patterns were observed in the mineralogical composition of this residue.

Residues from the Panamanian and Belizean corals include gibbsite, quartz, and several feldspars. These samples show that gibbsite (Al_2O_3) was introduced at the same time as the total amount of residue increased. The occurrence of gibbsite in the Panamanian specimen (Fig. 1) correlates in time with the start of a housing development in the Pearl Islands (P. W. Glynn, personal communication).

The type of noncarbonate detrital material in coral skeletons, therefore, may be dependent largely on spatial and temporal variations in the composition of sediment suspended in waters in which the corals lived. Thus, major changes in the kind of residue throughout the skeleton of a coral colony are probably the result of past changes in the character of suspended detritus.

Changes in the concentration and composition of trapped detritus should be included in baseline studies because suspended material is part of the total environment of a coral colony. Environmental interpretations could become more meaningful if mineralogical changes of trapped, suspended material were considered together with elemental distributions in coral skeletal carbonate. Detrital aluminosilicates are associated with many ecologically important trace elements¹⁵, so that any analytical scheme must consider the possible presence of detrital minerals if the calculation of partition coefficients is to be significant. Techniques for the separation of this detrital material must necessarily be chemical, because of its disperse nature in primary skeletal cavities. Furthermore, the techniques should be designed to prevent the release of adsorbed or included metal ions from the detrital minerals.

Previous elemental chemical analyses must be interpreted with caution in cases in which the presence of terrigenous detritus was not noted. Early coral-skeletal studies¹ concentrated on the major elements traditionally associated with the carbonate framework (Ca, Mg). A few trace metals were also investigated, but little real attention was paid to them until it was demonstrated¹⁶ that oxides of Fe, Al, and Si, in addition to most of the trace metals found in seawater, are present in coral skeletons. There have been attempts to calculate partition or distribution coefficients for these elements^{5,6}. Our work, and that of others¹⁶, suggests that most, if not all, Fe, Al, and Si, along with some alkalis and alkaline earths reported in coral

heads, are attributable to detrital aluminosilicates and other detrital minerals, rather than to material incorporated in the skeletal carbonate.

Coral samples were donated by P. W. Glynn, R. F. Perkins and F. T. Vernberg. We thank P. W. Glynn, J. W. Wells and W. A. Oliver for discussion and advice, and Mr W. R. Brown for operating the scanning electron microscope. Research was supported partially by Smithsonian Research Funds.

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Radiation dosage associated with ball lightning

A BALL lightning event occurred in a house in North Berkshire on May 8, 1970; here we report subsequent investigations using thermoluminescent dating techniques. According to some theories¹⁻⁴ matter close to the path of such an event may experience a radiation dose of order 1 to 1,000 rad. Since the house was built of brick and only 25 yr old, it seemed possible that the mineral inclusions in the brick would exhibit a level of thermoluminescence (TL) significantly in excess of the level resulting from the annual radiation dosage received from the uranium, thorium and potassium contained as impurities in the clay of the bricks themselves.

The material tested was supplied by Ashby and Whitehead⁵. Cores (mostly around 30 mm in length) were taken from bricks around the doorway through which the ball lightning is reported to have passed (cl, 2, 4 and 7 of Fig. 1) and a series of controls well displaced from the event were added to the collection (c3, 10 and 12 of Fig. 1). Radioactive analysis of the clay (by alpha counting in the case of uranium and thorium, and by flame photometry in the case of potassium) indicated

that those crystalline inclusions which are free of self-radioactivity, such as quartz⁵, would experience an annual dosage of a little under 0.5 rad, thus accumulating TL equivalent to 12 rad since the bricks were fired.

In the measurement procedure⁵ particular care was taken to suppress 'spurious' TL that was liable to show itself in the 275° C region of the glow curve; in addition to the utilisation of oxygen-free nitrogen, the grains were coated with silicone oil using an aerosol spray. The very low concentration of crystalline material in these bricks, (~ 1 to 2%), excluded the usual pretreatment of the sample by etching in hydrofluoric acid (this limits the sample to quartz and so ensures that the estimate of natural dose-rate is accurate). Radioactive analysis of an

Table 1 Measurements of equivalent doses in bricks from a North Berkshire house recorded as in the vicinity of a ball lightning event

	High temperature TL equivalent dose (rad)	Pre-dose TL equivalent dose (rad)
(i) Doorway cores		
c1 (i)	20	*
c2 (i)	16	*
c4.1 (i)	*	14
c4.2 (i)	14	*
c4.2 (ii)	16	*
c7.1 (i)	*	17
c7.1 (ii)	24	*
c7.2 (i)	19	*
c7.2 (ii)	*	15
c7.2 (iii)	*	16
(ii) Control cores		
c3 (i)	15	*
c3 (ii)	16	*
c10 (i)	19	*
c10 (ii)	*	12
c12 (i)	*	15
c12 (ii)	*	12

*Since some of the crystalline extracts were small only one method of TL analysis was attempted on each sample.

extract indicated that at least some of the crystalline minerals in the sample had an internal content of uranium and thorium even if the quartz present did not. A rough value of about 0.15 rad yr⁻¹ was estimated for the resulting extra annual dosage, adding a possible 3 rad to the calculated accumulated dose in the cores.

As well as the conventional high temperature TL analysis⁵ we investigated the sensitivity changes of the 100° C quartz peak^{6,7} under the combined effect of radiation plus heating to 500° C. A similar heating of freshly annealed geological quartz causes no sensitivity change, nor is there any change if the sample is irradiated and the TL induced allowed to decay away at room temperature (the storage half-life is about 145 min). But if the sample is heated to 500° C there is an increase in sensitivity which is proportional to the size of the 'pre-dose'—the radiation dose received between the annealing

and the heating to 500° C. In the present application we are concerned with the natural pre-dose that has been received by quartz in the brick between firing in 1945 and laboratory analysis in 1970.

The higher values of the estimated dose obtained by the high temperature method reflect the limitations on accuracy of this approach when only small amounts of sample are available (Table 1). The agreement between the predicted dose of 12 rad for quartz in the control cores and the observed pre-dose values (averaging at 13 rad) is satisfactory. The variations are probably due to the difficulties in estimation of the initial sensitivity values appropriate to quartz only. In the supposed 'hotter' doorway cores, though the average dose is slightly higher at 15.5 rad, the difference from the controls can hardly be treated as significant. If a ball lightning dosage occurred it would seem to be less than 5 rad in magnitude.

The model of ref. 1 would require a ball lightning event of less than 10⁶ J at 2 m to produce such a dose and the model of ref. 3 would require an energy content of less than 2 × 10³ J (less than 2 × 10⁻¹¹ of antimatter).

This work should not be treated as in agreement or otherwise with another TL study⁸ of possible effects of a ball lightning event in 1846. The author of ref. 8 was unable to detect any dosage in a damaged church steeple made of stone. Even the upper limit of 1,000 rad quoted earlier would be barely detectable in the face of the geological dose that the TL of such stone would record. We also feel that comments on the use of TL stored in the 110° C peak as an indicator of high dose radiation history should be read with the knowledge of the shortness of the peak's half-life. Even a delay of 1 d between event and TL measurement will reduce the peak intensity by a factor of around 1,000. But the sensitivity changes due to the pre-dose mechanism are stable over millennia.

We thank the Nuffield Foundation for support.

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Received May 28; revised July 5, 1974.

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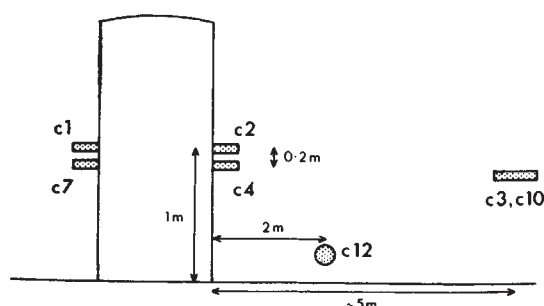


Fig. 1 Location of core samples.

New type of smectic mesophase?

4-Cyano-4'-octylbiphenyl (NC.C₆H₄.C₆H₄.C₈H₁₇) is a biphenyl derivative of considerable technological interest since it forms a colourless, stable smectic phase which exists at room temperature (21° C to 32.5° C). The corresponding ether 4-cyano-4'-octyloxybiphenyl (NC.C₆H₄.C₆H₄.OC₈H₁₇) forms a similar smectic phase at a higher temperature range (54.5° C to 67° C). Preliminary studies suggested that the smectic phases of these compounds are not miscible with any recognised type of smectic phase. Subsequent investigations (unpublished work by G.W.G.) have shown, however, that they are both miscible with a number of 'authentic' S_A phases.

The X-ray diffraction photographs of the smectic phases of the two biphenyls are similar and are of a novel type. They both consist of a sharp inner reflection and two diffuse outer reflections as shown in Fig. 1. All smectic phases previously studied

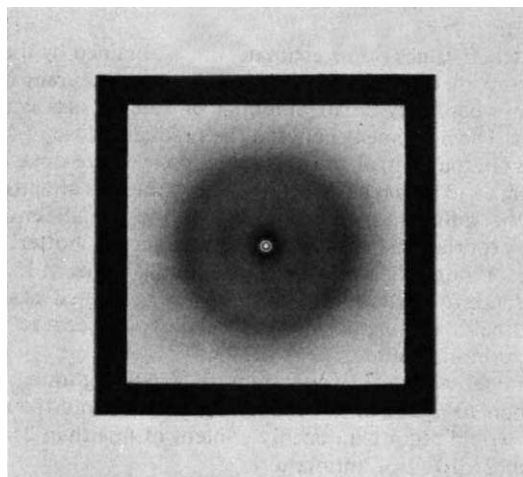


Fig. 1 The X-ray diffraction pattern of 4-cyano-4'-octylbiphenyl, taken at room temperature with a flat plate camera. The sample was contained in a Lindemann glass tube (of diameter 0.1 mm). No specific measures were taken to orientate the sample and the partial ordering apparent occurred spontaneously. The innermost sharp reflection corresponds to a repeat distance of 31 Å. The two diffuse reflections correspond to ranges of repeat distances centred on 8.6 Å and 4.3 Å.

have given X-ray diffraction patterns which fall into one of the three categories (listed by de Vries² as types α , β and γ). In all of these there are sharp inner rings corresponding to the smectic layer thickness. In type α patterns there is one diffuse outer reflection, in type β there is one sharp outer reflection and in type γ there are three sharp outer reflections.

In the diffraction pattern of the biphenyls, the outer diffuse reflection corresponds to a d spacing of 4.3 Å and is at more or less the same position as the single diffuse reflection given by previously studied S_A phases (all of which are essentially conjugated aromatic systems of the general type p -X.C₆H₄.A = B.C₆H₄.Y- p). We note that the diffuse reflection characteristic

of fluid hydrocarbon chains is found at a slightly lower angle, corresponding to a spacing of 4.5 Å or 4.6 Å, and no subsidiary maximum is discernible at this position in the diffraction pattern. The origin of the inner diffuse reflection is not apparent to us at this stage. The inner sharp reflection corresponds to a spacing of 31 Å, which is to be compared with the length of approximately 22 Å for the fully extended molecule. Some form of double layer structure is clearly mandatory. A number of possible models for the packing arrangement which have the correct layer thickness are shown diagrammatically in Fig. 2. All of these have layers of a sandwich construction. In model I the alkyl chains are fully extended and interdigitated. One questionable feature of this model is the juxtaposition of the CN groups. The bond dipole moments of these are appreciable (we would expect a value of about 4 debye units) and perhaps model II, which would reduce repulsive dipole-dipole interactions, is preferable. There is also the possibility incorporated into model III that the alkyl chains are disordered—which would reduce or remove the need for interdigitation. In the final model, IV, it is suggested that it is the interactions between the -CN groups, rather than between alkyl chains, which hold the two sheets of molecules together within the layer. It is hoped that this matter will be resolved (and the structural features which give rise to the two diffuse rings found) by our current investigations using mesophase samples oriented in a magnetic field and by extrapolating from the structures of the crystalline solids.

Optical studies by Gray and Harrison have indicated that both mesophases are uniaxial and only in the case of model IV are the individual layers uniaxial. Models I-III may, however, not be ruled out on this account because they would give uniaxial phases also if there were no correlation of the tilt directors from smectic layer to smectic layer.

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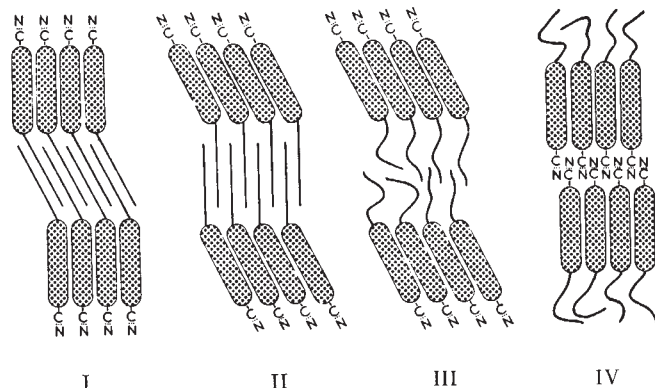


Fig. 2 Diagrammatic representations of possible alternative models for the smectic phases of cyanobiphenyl derivatives. I, Simple model featuring fully extended, interdigitated alkyl chains; II, model incorporating tilting of the biphenyl groups, which may be necessary to reduce the repulsive dipole-dipole interactions between the CN groups; III, model in which the alkyl chains are disordered and only partially interdigitated; IV, alternative model incorporating disordered alkyl chains where the two sheets of molecules in each layer are held together by the electrostatic interactions between the interdigitated CN groups in the centre of the layer.

Orientation perception by children

In a series of publications Bryant¹⁻³ has advanced the interesting idea that children's perceptual processes in the early years are characterised by an absence of absolute codes and an exclusive reliance on relative codes. Much of this work involves the perception of orientation of lines: in a successive discrimination task (that is one in which the choice stimuli are presented after the standard has been withdrawn) children below the age of 5 yr have been found able to distinguish horizontal from vertical lines but not oblique lines from their mirror-image. Bryant suggests that children adopt a binary match-mismatch code, which tells them whether lines parallel each other or not. Horizontal and vertical lines can be matched with the framework of the plaque on which they are presented and coded accordingly; oblique lines, however, merely produce a mismatch signal in such a comparison. Thus the young child cannot as yet code orientation in an absolute sense and instead requires the help of features of his environment with which the stimuli can be compared.

It follows from such a view that even oblique lines ought to be discriminated correctly by young children when the task is a simultaneous discrimination situation. In such a situation the standard is presented together with the choice cards, so that, according to the match-mismatch hypothesis, the child can code the orientation of the correct line as being parallel with the standard. In simultaneous comparisons, near-perfect performance should therefore occur, even when the discrimination involves oblique lines. Bryant's studies seem to confirm this; we have, on the other hand, not been unable to replicate his findings.

Our data were obtained by four groups of postgraduate students in successive years between 1970 and 1973, using identical methods and thus representing four replications. The 114 subjects (mean age 4 yr 5 months) were seen individually in a room at the nursery school which they attended, and presented with 4 inch square white cards on which 3 inch long black lines were drawn. There were two stimulus conditions, involving discrimination between a horizontal and a vertical line and between two oblique lines respectively, and two test conditions, that is, simultaneous and successive discrimination. Subjects served under all four conditions, receiving eight trials under each.

An analysis of variance of the error scores obtained by the whole group shows highly significant effects for stimuli ($F = 88.68$, $P < 0.001$), for test conditions ($F = 69.96$, $P < 0.001$), and for the interaction between these two terms ($F = 24.58$, $P < 0.001$). It seems that the children found the horizontal-vertical discrimination considerably easier than that between oblique lines; that they performed very much better in the simultaneous than in the successive discrimination test; and finally, and most important, that the oblique stimuli were difficult under both conditions (see Table 1).

Table 1 Mean number of errors (max. 8, chance level 4)

Stimuli	Simultaneous task		Successive task	
	Mean	s.d.	Mean	s.d.
Horizontal-vertical	1.10	1.48	3.10	1.80
Obliques	3.17	2.01	3.89	1.59

It is thus apparent that even when the standard card and the two choice cards were presented at the same time, the children were unable to discriminate among obliques. The presence of a stimulus for matching purposes was of little help in judging similarity, whereas no such difficulty arose in the discrimination of horizontal and vertical lines. Exactly the same pattern of results emerges when each of the four sets of data obtained in different years is analysed separately.

These results thus fail to confirm those reported by Bryant. It is not clear what factors may be responsible for the discrepancy, but taken in conjunction with the failure by Fellows and Brooks⁴ to replicate another deduction from Bryant's theory, namely that children should be able to discriminate obliques in a successive task when the lines are presented within a diamond-shaped framework, it becomes apparent that reservations are necessary in accepting the match-mismatch explanation. It seems rather more likely that children initially have an absolute difficulty in coding oblique lines that is not overcome by the provision of parallel lines in the environment.

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Diapause potential in tropical flesh flies

FLESH flies (Diptera: Sarcophagidae) of the temperate region use the short, cool days of late summer and early autumn as a reliable cue to signal the advent of winter^{1,2}. Flies may enter an overwintering pupal diapause in August, and the adults will not emerge until the following May³. Tropical flies, by contrast, are not exposed to a regularly occurring season of lethal temperatures, and flesh flies from Nairobi, Kenya, have been reared outside throughout the year without the occurrence of diapause (D. L. D., unpublished). Yet four species from equatorial Africa that have been examined, *Sarcophaga inzi*, *S. monospila*, *Poecilometopa punctipennis* and *P. spilogaster*, have all demonstrated the potential to enter pupal diapause. During diapause in both the temperate^{1,2} and tropical species, pupal development is halted at the early phaenoccephalic stage.

Colonies of experimental flies originated from wild females, and diapause experiments were carried out within the first year of laboratory rearing. Larvae from single females were reared in separate packets of beef liver¹. *P. spilogaster*, a species collected in Nairobi (1°S), was examined most extensively.

Induction of diapause is temperature dependent. If the larvae and pupae of *P. spilogaster* are maintained at temperatures of 20° C or more, development proceeds without interruption (Fig. 1a). At 18° C, a few pupae enter diapause, and as the temperature decreases the incidence of diapause increases. Diapause is not, however, an immediate reaction to the temperature experienced by the phaenoccephalic pupa. The transfer of young pupae to low temperature does not result in diapause. The ability to enter diapause is programmed during larval life. Maximum diapause response is obtained by cold exposure throughout larval life. A decrease in the duration of cold exposure (15° C) received by the larva decreases the incidence of diapause (Fig. 1b). If more than the first 2 d of larval life are spent at 25° C, the diapause response is reduced to less than half the maximal response. During July and August, the coldest months in Nairobi, mean daily temperatures are frequently as low as 15–18° C; however, for induction of diapause a series of 1–2 weeks of cool days would have to occur in direct succession. An interruption by several warm days could avert the diapause. Although an accumulation of sufficient cold days for diapause induction occurs only rarely in Nairobi, at higher elevations within 20 km of Nairobi, conditions that could induce diapause occur annually.

Unlike the temperate species, which react strongly to photoperiod², the tropical species seem to be uninfluenced by daylength for diapause induction. Differences could

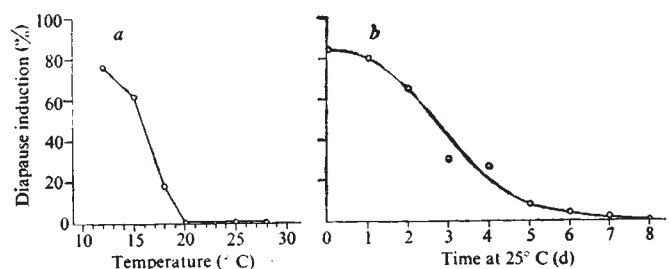


Fig. 1 a, Incidence of pupal diapause in *P. spilogaster* that were reared throughout larval life at different temperatures and a daily 12-h photophase. Each point represents 982–1,411 pupae. b, Incidence of pupal diapause in *P. spilogaster* that were held at 25° C for different periods following larviposition and then transferred to 15° C. Each point represents 174–214 pupae.

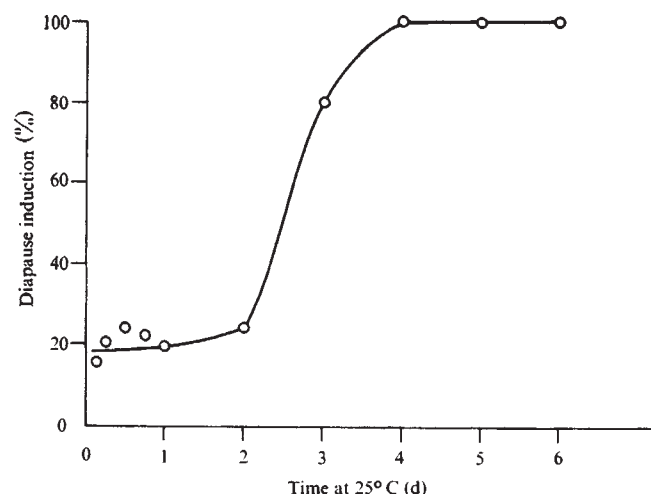


Fig. 2 Percentage of *P. spilogaster* that terminate diapause when transferred from 15°C to 25°C for different periods of time and then returned to 15°C. Pupae were in diapause 10 d before exposure to 25°C. Each point represents 25 pupae.

not be established among flies reared under daily photoperiods of 1, 10, 11, 12, 12.25, 12.50, 13, 15 and 24 h. Since seasonal variation in daylength is only 7 min in Nairobi, the results are not surprising.

Termination of diapause is temperature dependent in both temperate² and tropical flesh flies; spontaneous termination of diapause occurs later at lower temperatures. At 18°C, diapause in *P. spilogaster* lasts 72.6 ± 2.9 d (mean $\pm$ s.e., $n=108$), at 15°C 100.9 ± 2.2 d ($n=268$), and at 12°C 234.6 ± 6.8 d ($n=207$). Flies from the two regions, however, do differ greatly in their rate of response to favourable temperatures. If young diapause pupae of a species from a temperate region are transferred to 25°C, more than 100 d may elapse before diapause is terminated². If diapause pupae of *P. spilogaster* are transferred from 15°C to 25°C, adult development can be observed within 3 d. The amount of exposure to 25°C required for diapause termination for *P. spilogaster* is seen in Fig. 2. In this experiment, pupae were exposed to 25°C for varying periods of time and were then returned to 15°C; 4 d at 25°C was sufficient for diapause termination in all pupae. The difference in the ability to terminate diapause between flies of the two regions is a reflection of the environmental conditions to which each is exposed. The temperate region winter is a predictable event of long duration. The tropical seasons are less predictable. Years may go by without the species entering diapause. If the fly does enter diapause during a brief cold period, it is not committed to a long period of dormancy and can react immediately to a stimulus of higher temperatures.

That diapause has been observed in all four Sarcophaginae examined suggests that the potential for diapause may be common among the tropical species. The Sarcophaginae are thought to have originated in the tropics and subsequently spread to the temperate regions³. The necessity for diapause increases with distance away from the equator, and the temperature dependency of diapause becomes supplemented with photoperiodic cues in the temperate region.

I thank Professor Jan de Wilde for his enthusiastic support and NIH-NIAID for a postdoctoral fellowship.

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A fly's leap from paralysis

THE first temperature-sensitive paralytic mutant of *Drosophila melanogaster*, *para*^{ts}, was isolated four years ago^{1–3}. The mutation was mapped as a point at 53.9 on the X chromosome⁴. The present day stock of *para*^{ts} moves normally at 22°C, but is paralysed within 10 s at 29.5°C. Paralysis is usually complete. The legs are held close to the body causing the fly to lie on its back or side. When returned to 22°C the fly recovers its mobility within 5 s. A much slower and less complete recovery occurs when the flies are left at 29.5°C (ref. 4). Only after 30 min are they able to walk and after 2 h a few flies can be induced to fly by shaking their container. The mutation has been shown to act in a regionally autonomous fashion within the head and thorax⁵.

By genetic and pharmacological means, activity can be elicited from *para*^{ts} flies at temperatures which would otherwise paralyse them. Augmented inhibition is suggested as a possible cause for this type of paralysis.

Our work concerns the interaction of *para*^{ts} with *Hyperkinetic*¹ (*Hk*¹), another sex-linked mutation and the pertinent behaviour of this mutant is its response to movements nearby⁶. When waved at by hand it usually jumps and often tumbles. In our laboratory, Patrick Wong discovered that the fly responds similarly to a high intensity, high frequency stroboscopic light⁷. High speed motion picture films show that the take off and flight are abnormal⁸.

While hand waving seems to elicit a few jumps from *Hk*¹*para*^{ts} flies at 22°C, these double mutants do not respond to this stimulus after they have been paralysed at 29.5°C. *Hk*¹*para*^{ts} flies, however, usually respond to the strobe light at 22°C and a few jumps can even be elicited after they have been paralysed at 29.5°C.

*Hk*¹*para*^{ts} flies did not respond under the high levels of heat and light emitted by the lamps used for high speed photography. Fortunately, flies carrying mutations which produce yellow cuticle (*y*) and white eyes (*w*) (ref. 9) together with *Hk*¹ and *para*^{ts} (that is, *y w Hk*¹*para*^{ts}) were very responsive. Figure 1 is a photographic sequence of a *y w Hk*¹*para*^{ts} fly responding to a flashing light at 29.5°C. The first frame shows the paralysed fly lying on its right side facing away from the camera. After the flash, the fly pushes off with the midlegs and beats its wings twice before leaving the field of view. Other sequences show as many as seven wing beats. Normally *Hk*¹*para*^{ts} and *y w Hk*¹*para*^{ts} flies recover their mobility over a prolonged period, just as *para*^{ts} flies do. Yet at each instant from the time that paralysis is first induced, these flies retain the competence to see and to respond.

The light-induced escape from paralysis is short lived. The fly travels 2 or 3 cm and falls down in a paralysed state again. Furthermore, the response is often incomplete. The fly may push off with its midlegs, simultaneously lifting and spreading its wings only to return its wings to a resting position in midflight. The extension of the midlegs may also be incomplete: the femurs extending while the tibiae remain folded. Such blocks in the jump and flight response have not been seen in wild type or *Hyperkinetic* flies and are therefore probably caused by the *para*^{ts} mutation.

Table 1 shows that the capacity of the *Hk*¹ mutation to make the *para*^{ts} mutant jump at high temperature is greatly enhanced by the *w* mutation¹⁰. *w Hk*¹*para*^{ts} flies respond to both flashing

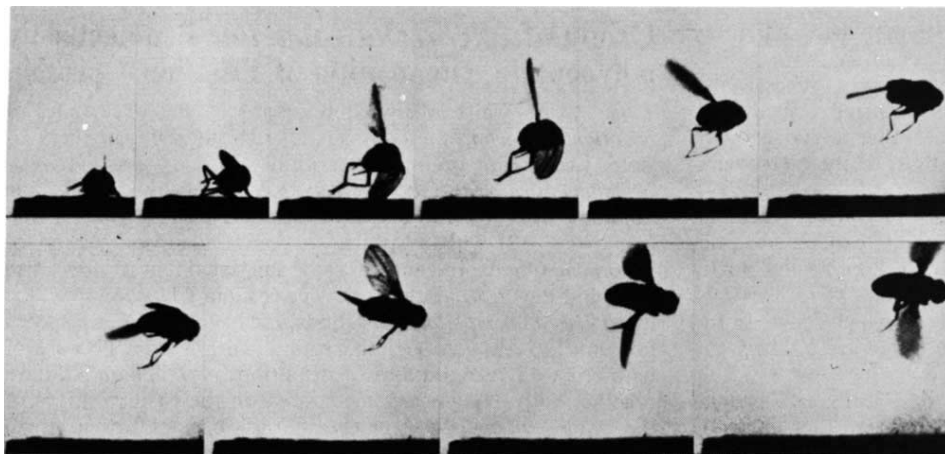


Fig. 1 The response of a $y w Hk^1 para^{ts}/Y$ fly to flashing light at 33°C. The sequence was recorded at 400 frames s^{-1} in 5,500 lux illumination. 100 light flashes s^{-1} were delivered by a Grass PS2 photostimulator at intensity setting '8'. The PST2 lamp and reflector were placed 20 cm from the fly. Paralysis was complete before stimulation.

light and movements. There is no indication that the mobility of $w Hk^1 para^{ts}$ flies is greater than that of $para^{ts}$ flies at elevated temperatures. Rather the added mutations seem only to sensitise the flies to visual stimuli.

Obviously, the sensory, neuronal and muscular elements involved in seeing, jumping and flying are functional. A degree of motor competence in paralysed $para^{ts}$ flies has also been demonstrated previously by the production of leg jerks through electrical stimulation of the cervical connectives¹¹. On the other hand, the temperature-induced paralysis may be caused by abnormally high levels of inhibition of elements innervated by inhibitory neurones. When the light reaching the sensory cells is increased by the w mutation¹², and the motor system is genetically primed by the *Hyperkinetic* mutation, certain visual stimuli elicit strong signals which override inhibition and call forth the high priority response of escape⁸.

Picrotoxin specifically blocks the action of γ -aminobutyric acid¹³, a probable inhibitory transmitter in insects¹⁴⁻¹⁶. Thus it may also prevent temperature-induced paralysis from occurring in $para^{ts}$ flies.

Injections of picrotoxin elicited a random activity in $para^{ts}$ and wild type flies at 22°C. Low concentrations caused the normal activities of the flies to be carried out in a shakey, uncoordinated manner. Higher concentrations increased the activity of the fly and abolished all coordination. The behaviour of all flies was not identical, but in various individuals, movements of the head, antennae, proboscis, wings, halteres,

Table 1 Average response of all mutant combinations of y , w , Hk^1 and $para^{ts}$ to hand waving and flashing light

Genotype	Number of jump responses $\pm$ s.d. to 50 stimuli					
	22 $\pm$ 1°C			29.5 $\pm$ 0.5°C		
	hand waving	light intensities '4'	'8'	hand waving	light intensities '4'	'8'
$y w Hk^1 para^{ts}$	22 $\pm$ 10	32 $\pm$ 12	15 $\pm$ 5	17 $\pm$ 13	36 $\pm$ 11	27 $\pm$ 12
$+ w Hk^1 para^{ts}$	31 $\pm$ 17	42 $\pm$ 13	19 $\pm$ 11	20 $\pm$ 19	35 $\pm$ 17	34 $\pm$ 20
$+ + Hk^1 para^{ts}$	3 $\pm$ 4	23 $\pm$ 13	32 $\pm$ 14	0 $\pm$ 1	3 $\pm$ 4	5 $\pm$ 6
$+ + Hk^1 +$	41 $\pm$ 7	22 $\pm$ 13	29 $\pm$ 11	35 $\pm$ 9	3 $\pm$ 3	3 $\pm$ 3
$+ w Hk^1 +$	43 $\pm$ 3	47 $\pm$ 3	47 $\pm$ 2	48 $\pm$ 2	49 $\pm$ 2	48 $\pm$ 2
$y w Hk^1 +$	35 $\pm$ 11	44 $\pm$ 7	24 $\pm$ 13	47 $\pm$ 3	46 $\pm$ 4	47 $\pm$ 3
$y w + +$	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$y + + +$	0 $\pm$ 1	6 $\pm$ 7	12 $\pm$ 12	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$+ w + +$	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$+ + + para^{ts}$	1 $\pm$ 1	5 $\pm$ 6	8 $\pm$ 9	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$y + + para^{ts}$	0 $\pm$ 0	10 $\pm$ 12	9 $\pm$ 11	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$+ w + para^{ts}$	0 $\pm$ 0	5 $\pm$ 7	1 $\pm$ 2	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$y + Hk^1 +$	45 $\pm$ 6	39 $\pm$ 11	40 $\pm$ 12	36 $\pm$ 14	28 $\pm$ 19	22 $\pm$ 14
$y + Hk^1 para^{ts}$	5 $\pm$ 10	36 $\pm$ 15	39 $\pm$ 15	0 $\pm$ 0	3 $\pm$ 6	5 $\pm$ 5
$y w + para^{ts}$	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
$+ + + +$	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
(Canton-S)						
$+ + + +$	0 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
(Oregon-R)						

Ten males, 4-5 d old, of each genotype, were placed individually into 8 cm $\times$ 2 cm vials, which were then laid on a white background. Each fly was subjected to quick horizontal passes of the hand about 5 cm above the vial, or light stimuli, each consisting of 10 flashes of 0.01 s duration, produced by a Grass PS2 photostimulator. At settings '4' and '8', the peak luminous intensities of the xenon lamp are stated to be about 2.2×10^5 and 2.4×10^5 candlepower. The lamp and reflector were placed 15 cm above the vial. Ambient intensities of illumination from overhead fluorescent fixtures were 700 lux at 22 $\pm$ 1°C and 350 lux at 29.5 $\pm$ 0.5°C.

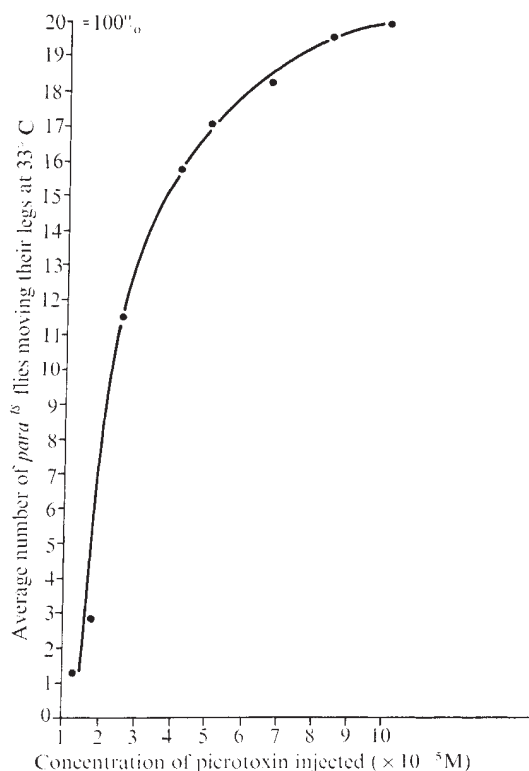


Fig. 2 Effect of picrotoxin on leg movements of $para^{ts}$ flies at 33°C. Male and female $para^{ts}$ flies, 3-24 h old, reared at 17°C were immobilised on a cold plate¹⁸ and injected in the ventral posterior abdomen with about 0.25 μ l of picrotoxin in *Drosophila* Ringer's¹⁹ or *Drosophila* Ringer's alone. The dissecting scope lights were fitted with small water chambers to reduce heat. Uninjected control flies were kept cold for the same period. Two lots of 10 injected and 10 uninjected flies were placed in four covered thin plastic dishes²⁰. At 22°C they recovered from the cold until at least 80% of the uninjected flies were able to stand (4-6 min). The dishes were then transferred to a water heated observation chamber. The interior of each dish rose from 22°C to 33°C within 220 s. From 250-600 s, the number of flies able to move even a leg segment was noted at 50 s intervals. Other types of movements were not monitored. The average number of flies moving their legs is recorded on the graph. Over 90% of uninjected and Ringer's injected flies lay in a state of paralysis. In the same conditions, wild type *Oregon-R* flies were not paralysed, but did tend to stand still, walking and preening infrequently.

abdomen and legs were seen. We assume that these activities were the result of a blanket disinhibition of many parts of the nervous system which generate motor activity. These uncoordinated movements of *para*^{ts} also occurred, albeit less vigorously, at 33° C. The number of flies able to move their legs increased with increasing concentrations of the picrotoxin injected (Fig. 2).

In our experiments, the concentration of picrotoxin reaching the central and peripheral nervous system are expected to fall within the range which has been shown to specifically block GABA-mediated peripheral inhibition (5×10^{-6} M to 4×10^{-5} M) (ref. 13). Other physiological effects which have been reported¹⁷ in the 5×10^{-5} M to 10^{-2} M range are unlikely to account for the behaviour we observed.

Picrotoxin injections caused as many as 8 deaths in a group of 20 treated flies. Flies which survived recovered their normal behaviour on the day following treatment.

The motor elements of the paralysed fly seem to be functional but the possibility that paralysis may be caused by the reduced excitability of certain motor elements cannot be ruled out. In these circumstances, normal levels of inhibition may cause paralysis, while the blocking of normal inhibition may have caused the movements that we observed. On the other hand, augmented inhibition still provides a reasonable explanation for a paralysis which can be overridden or disinhibited.

This work was supported by a grant to W.D.K. from the United States Public Health Service. We thank Barry Hanstein for the photography, the D.B. Milliken Company of Arcadia, California, for the loan of a high speed motion picture camera and Kazuo Ikeda, William Trout III and Patrick Wong for comments on the manuscript.

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Origin of *Nicotiana tabacum* L. detected by polypeptide composition of Fraction I protein

THERE is no well-authenticated record of the occurrence of *Nicotiana tabacum*, the commercial tobacco plant, in the wild state, and so its origin and evolution are of great interest. *N. tabacum* ($n=24$) is believed to have arisen by chromosome doubling after hybridisation of *N. sylvestris* Spegazzini and Comes ($n=12$) with a species in the Tomentosae section of *Nicotiana*. Goodspeed and Clausen¹ suggested that *N. tomentosa* Ruiz and Pavon ($n=12$) was the species, but Clausen² amended it to *N. tomentosiformis* Goodspeed ($n=12$). Goodspeed³ favoured *N. otophora* Grisebach ($n=12$), however, because of its present-day geographical distribution; *N. otophora* is found together with *N. sylvestris* in an area on the eastern slopes of the Andes whereas *N. tomentosiformis* occurs further north where *N. sylvestris* has not been found. Later evidence, however, suggests *N. tomentosiformis* as the more likely progenitor of *N. tabacum*. Gerstel⁴, from an analysis of the segregation of artificial polyploids of *N. tabacum* × *N. tomentosiformis* and *N. tabacum* × *N. otophora*, concluded that *N. tomentosiformis* showed the greater chromosome homology with *N. tabacum*, and Sheen⁵⁻⁷ from an analysis of isoenzyme patterns and sterol composition supported the conclusion that *N. sylvestris* and *N. tomentosiformis* were the likely progenitors of *N. tabacum*. From an analysis by isoelectric focusing of the polypeptide composition of Fraction I protein isolated from *N. tabacum* and the putative progenitor species, we now report that *N. tabacum* arose from the hybridisation of *N. sylvestris* ♀ × *N. tomentosiformis* ♂.

Fraction I protein (ribulose 1,5-diphosphate carboxylase) consists of two kinds of subunits which differ in size. The large subunits are coded by chloroplast DNA⁸ whereas the small subunits are coded by nuclear DNA⁹. By isoelectric focusing in 8 M urea the large subunit is resolved into three polypeptides and the small subunit into one or more polypeptides¹⁰. The patterns obtained are characteristic of each species of *Nicotiana*; no variation of pattern has been observed within any species although many plants from several seed collections have been examined. The polypeptide patterns for Fraction I

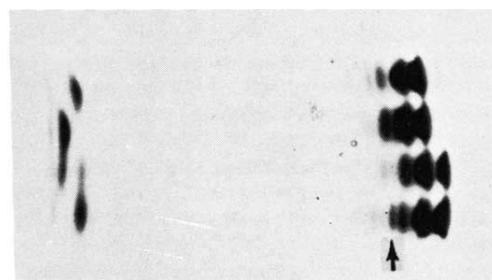


Fig. 1 Polypeptide composition of Fraction I proteins from *N. tabacum* and the putative progenitor species. Crystalline Fraction I protein was dissolved in 8 M urea-0.5 M Tris-HCl, pH 8.5 and reduced by the addition of dithiothreitol. The protein was S-carboxymethylated by the addition of iodoacetic acid, and after 15 min excess iodoacetic acid was removed by passage through a 1×7 cm column of Sephadex G-25 (coarse). Protein (20 µg per sample) was then applied to a prefocused 4.5% polyacrylamide gel slab containing 1% Ampholine, pH 5-7, and 8 M urea. Isoelectric focusing was performed for 18 h at 300V. Polypeptide bands were stained with bromophenol blue. Minor bands marked by arrows are artefacts arising by carbamylation of the protein by cyanate present in urea solutions¹⁰. Samples, from left to right, were *N. otophora*, *N. tomentosiformis*, *N. tabacum* and *N. sylvestris*. Large subunit polypeptides, pH 6.5; small subunit polypeptides, pH 6.0.

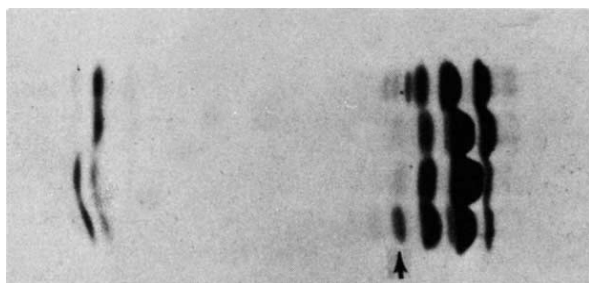


Fig. 2 Polypeptide composition of Fraction I proteins. Fraction I proteins were S-carboxymethylated and subjected to isoelectric focusing as described for Fig. 1. Arrows indicate artefacts due to carbamylation of the protein. Band splitting is due to the reaction of polyphenols with the protein during isolation (J.C.G., S.D.K. and S.G.W., unpublished results). All samples were 20 µg protein. Samples, from left to right, were *N. sylvestris* × *N. otophora* ($n=24$), *N. sylvestris*, *N. tabacum*, *N. sylvestris* × *N. tomentosiformis* ($n=24$).

protein isolated from seven different cultivars and a polyploid series of *N. tabacum* were all similar (K. Chen, S.D.K., J.C.G. and S.G.W., unpublished results). Isoelectric focusing of Fraction I protein therefore readily provides phenotypic markers for both the nuclear and chloroplast genomes¹¹.

Crystalline Fraction I protein was prepared from young leaves by a simple method¹². Modification of the homogenisation buffer was necessary to obtain crystals from *N. tomentosiformis* and *N. otophora*; Dowex 1-(×2) (20% w/v), KCN (1 mM), sodium metabisulphite (10 mM) and bovine serum albumin (0.1% w/v) were included in the homogenisation buffer to prevent interaction of the protein with polyphenols¹³. The Fraction I proteins were recrystallised three times before analysis of the polypeptide compositions by isoelectric focusing.

The polypeptide compositions of Fraction I proteins isolated from *N. tabacum* and its putative progenitor species are shown in Fig. 1. The protein from *N. tabacum* is composed of three polypeptides in the large subunit and two polypeptides in the small subunit. Comparison with the polypeptide compositions of Fraction I protein from the other three species indicates that *N. sylvestris* contributed the large subunit polypeptides and therefore was the maternal parent in the original hybridisation. *N. sylvestris* also contributed one of the two small subunit polypeptides of *N. tabacum*, the other polypeptide being contributed by *N. tomentosiformis*. This analysis indicates that the original hybridisation was *N. sylvestris* ♀ × *N. tomentosiformis* ♂ and that *N. otophora* was not involved in the origin of *N. tabacum*.

To further verify this conclusion Fraction I proteins were isolated from experimentally-produced hybrid plants and the polypeptide compositions were analysed by isoelectric focusing. As shown in Fig. 2, the polypeptide composition of Fraction I protein isolated from the amphidiploid ($n=24$) of *N. sylvestris* × *N. tomentosiformis* is exactly the same as the composition of the protein from *N. tabacum*, whereas the Fraction I protein from the amphidiploid ($n=24$) of *N. sylvestris* × *N. otophora* has a different small subunit composition from the protein from *N. tabacum*. The amphidiploid of *N. sylvestris* × *N. tomentosiformis* is taxonomically indistinguishable from *N. tabacum* and is readily crossed with tobacco cultivars.

The analysis of Fraction I proteins therefore provides a convenient means for determining the origin of plant species that have arisen by interspecific hybridisation. The particular advantage in the use of Fraction I protein is that, because the large subunit polypeptides are inherited solely from the

maternal parent, it is possible to determine the exact parentage in the original hybridisation.

The multiple polypeptide compositions of the large and small subunits of Fraction I proteins have also been demonstrated in the proteins isolated by conventional chromatographic methods from spinach (*Spinacia oleracea*), soybean (*Glycine max*) and barley (*Hordeum vulgare*) (K. Chen, S.D.K., J.C.G. and S.G.W., unpublished results). It therefore seems probable that the method used with the *Nicotiana* species can be applied to studies of the origin of species in other plant families.

This work was supported by grants to S.G.W. from the National Institutes of Health, US Public Health Service and from the US Atomic Energy Commission. We thank Dr L. G. Burk, US Department of Agriculture, Oxford Tobacco Research Station, for seeds of the amphiploid *N. sylvestris* × *N. tomentosiformis*.

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Received June 24; revised September 3, 1974.

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Lithium treatment reduces morphine self-administration in addict rats

RECENT reports suggest that opiate-seeking behaviour in experimentally-addicted animals can be reduced by administering other drugs, such as α -methyl-*p*-tyrosine and haloperidol^{1–4}; and that haloperidol can also alleviate symptoms of heroin withdrawal in man⁵. Both drugs have been shown to reduce morphine-induced hyperactivity in mice⁶.

Lithium also has been shown to interact with morphine: it can sometimes reduce morphine-induced hyperactivity in mice^{7,8} and potentiate morphine analgesia in rats⁹. We have therefore investigated the possibility that lithium may affect the amount of voluntary ingestion of morphine by addict rats.

Naive rats normally reject solutions of morphine, presumably because of their bitter taste, but with a suitable drinking schedule they eventually learn to consume appreciable amounts^{10,11}, especially if the morphine is given in solutions of sucrose¹².

Male hooded rats, weighing 180–200 g at the beginning of the experiment and singly housed, were first trained to satisfy their daily fluid requirements during a limited time (1200h–1500h)

with food being available *ad libitum*. We have shown that rats readily adapt to this schedule and maintain apparent good health for at least 12 months¹³.

The rats were then made dependent on morphine by being given bottles containing solutions of 10% w/v sucrose + 0.5 mg ml⁻¹ morphine hydrochloride (SM) as their only source of fluid. Within a few days they learned to drink more of this solution than they had previously drunk of water. To test the strength of their preference for SM the animals were, after 6 weeks of this forced SM drinking, given daily choices between two bottles which contained either SM or a solution of 10% sucrose alone (S). On average the rats drank some 57% of their total fluid intake in the form of SM. There were considerable differences among them but individual rats were remarkably consistent over long periods of time. (Three out of the seventeen rats were excluded from the experiment as they persistently rejected the SM; nevertheless the statistical comparisons reported here remained significant even with the three rats included in the analyses.)

Fluid consumption was measured throughout by weighing the drinking bottles and applying a correction for spillage in the course of placing and removing bottles from the cages. The positions of the bottles (right or left) were varied randomly each day to allow for possible positional preferences. Statistical comparisons are based on two-tailed *t* tests and on analyses of variance designed to take into account repeated measures on the same subjects¹⁴.

After 3 weeks of daily SM/S choices, the rats were prepared for lithium administration by being weighed and injected intraperitoneally with 13 ml kg⁻¹ of isotonic saline 3 h before the beginning of their daily drinking period for 2 weeks. Then half the rats were injected intraperitoneally with 2 mmol kg⁻¹ of an isotonic LiCl solution and half continued being injected with saline. The particular dose of LiCl was chosen because our previous experiments had shown it to be behaviourally effective and without manifest toxic effects¹⁵, whilst yielding plasma levels approximating the lower end of the therapeutic range in man¹⁶. As lithium efflux from the brain is rather slow it is likely that our schedule of injections was adequate for maintaining fairly constant brain Li levels¹⁷.

After the very first injection of LiCl, the rats reduced their normal intake of SM from 23 ml d⁻¹ to 15 ml d⁻¹, that is, by approximately 35%, and increased the intake of S by 47% from 15 to 22 ml d⁻¹ ($P < 0.05$). This pattern of drinking

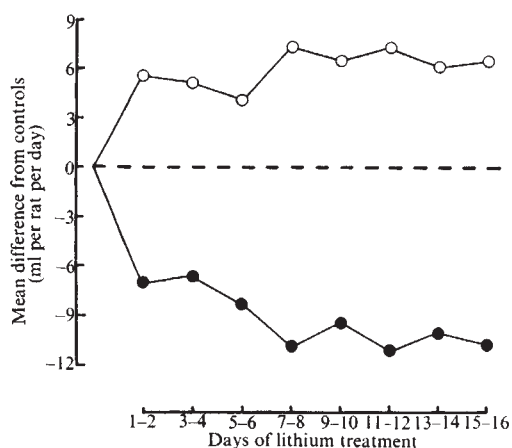


Fig. 1 Addicted rats which were given daily choices between solutions of, ●, 10% sucrose + 0.5 mg ml⁻¹ morphine hydrochloride (sucrose-morphine) and, ○, 10% sucrose alone (sucrose), were injected daily with either 13 ml kg⁻¹ of isotonic saline or with 2 mmol kg⁻¹ of LiCl ($n = 7$ per group). Lithium-treated rats reduced their consumption of sucrose-morphine from 23 ml d⁻¹ to an average of 15 ml d⁻¹, saline controls (---) increased theirs slightly. The results are represented as mean differences from controls.

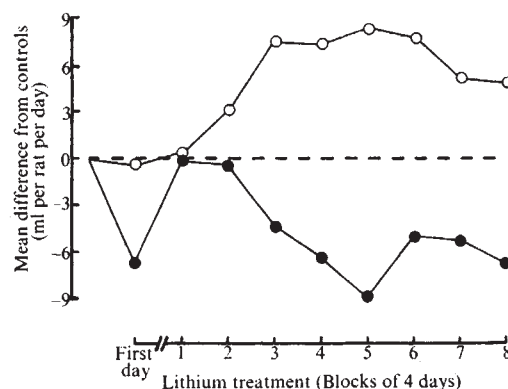


Fig. 2 Addicted rats which were given daily choices between sucrose-morphine (●) (Fig. 1) and tap water (○) were injected with 2 mmol kg⁻¹ of isotonic LiCl ($n = 9$) or 13 ml kg⁻¹ of saline ($n = 8$). Lithium treated rats reduced their sucrose-morphine consumption consistently only after some 12 days. The results are presented as differences between LiCl and saline controls (---).

persisted relatively unchanged throughout the 16 d of lithium treatment (Fig. 1). Lithium-treated rats consistently drank less SM ($F = 10.5$, $d.f. = 1$ and 12, $P < 0.01$) and more S ($F = 5.5$, $P < 0.05$).

The possibility could not be excluded that these changes in the relative amounts of the two fluids consumed might have been the result merely of an interaction of lithium with taste, that is, lithium (injected intraperitoneally) could have increased the bitterness (and thus the aversiveness) of the SM, or it could have increased the relative palatability of the S. Therefore, in a second experiment, 24 rats, after being accustomed to satisfying their thirst during the 3 h periods each day, were divided randomly into two groups. Twelve rats were given solutions of 10% sucrose + 0.025% quinine hydrochloride¹¹ (SQ) and twelve 10% sucrose solution alone as their sole source of fluid for 5 weeks. This concentration of quinine proved, initially, to be as aversive as the SM at the beginning of the previous experiment. When the SQ rats were subsequently given daily choices between SQ and S, they persistently drank some 40% of their daily fluid intake in the form of SQ. The remaining twelve rats which had been given sucrose alone to drink continued to receive S only. All rats were then handled and injected with saline for 2 weeks, and as in the previous experiment, they were divided into equal groups which continued to be injected with saline or were given 2 mmol kg⁻¹ isotonic LiCl for 2 d. Lithium treatment had no effect on the amounts of either SQ or S consumed, regardless of whether the rats had a choice between SQ and S, or were given S alone.

These results suggest, therefore, that the reductions in the amount of morphine ingested during lithium in the SM experiment were unlikely to have resulted merely from changes in the palatability of the fluids, as in the SQ experiment lithium did not alter the consumption of either S or SQ.

To analyse the lithium-morphine interactions further, an experiment was devised in which the rats would voluntarily consume much greater amounts of morphine (44 mg kg⁻¹ d⁻¹) than in the original experiment (27 mg kg⁻¹, $P < 0.001$). This was achieved by training 17 rats to drink SM in a way similar to that used in the first experiment, but in this instance the rats were given a choice between SM and plain tap water. They consequently drank more than 90% of their total fluid requirements in the form of SM. When, at the appropriate stage of the experiment, they were injected with 2 mmol kg⁻¹ LiCl their SM consumption was reduced ($F = 29.7$, $d.f. = 1$ and 15, $P < 0.01$) and their consumption of water increased ($F = 29.7$, $P < 0.001$); however, in these rats the reduction of morphine intake became consistent only after some 12 d (Fig. 2), possibly because the degree of their dependence was based on a much higher dose of morphine.

It seems fair to conclude that we have clearly demonstrated a robust interaction between morphine and lithium substantial reductions of morphine self-administration were obtained in two different experiments. Should further research establish the generality of these results, their possible implications for clinical practice seem obvious.

We thank David Blundell for help with the experiments, Elizabeth Paul for help with statistics, Dr D. H. Jenkinson and Professors J. W. Black, A. Graham and H. O. Schild for comments and the Medical Research Council and the Foundations Fund for Research in Psychiatry for support.

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Received July 25, 1974.

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Experience modifies morphine-induced behavioural excitation of mice

In many animal species, relatively low doses of morphine produce not only analgesia but also a state of behavioural excitation, restlessness and increased locomotor activity which has been defined as 'running fit'. A large intra-specific variability in locomotor activity has been recorded after the same dose of opiates¹ and clear strain differences have also been reported. Morphine for example, has been shown to enhance the locomotor activity of C57BL/6J mice and to drastically reduce it in the DBA/2J strain².

In man a number of studies indicate that the mechanisms responsible for individual differences in the reactivity to different psychotropic agents such as tranquilisers or hallucinogens are under genetic control^{3,4}. A number of environmental, social and psychological factors, however, may also play a role in the determination of the effects of many psychotropic agents such as psychedelic drugs or opiates⁵ and it is generally acknowledged that the social environment influences the action of centrally acting drugs⁶. These factors have been scarcely studied in animals but a number of experiments demonstrate that drug action is affected by group interactions⁷ or by the environment⁸. In particular, Steinberg and her associates⁹ investigating maze exploratory behaviour in individual rats, have shown that

past experience can have a very marked effect on, or even abolish the increase in, activity which normally results from different drugs or drug mixtures.

Our studies were designed to see if the stimulating effects of morphine on behaviour are determined, in addition to genetic mechanisms, also by environmental factors. C57BL/6J mice were used in this experiment as this strain was shown to be particularly reactive to the stimulating effects of morphine on locomotor activity as measured by a procedure previously described¹⁰. We first tested the effects of a single morphine injection on the motor activity of eight naive mice during a 25 min session. Morphine-HCl was injected intraperitoneally at a dose of 20 mg kg⁻¹, which was found to exert a clear effect on motor behaviour in this strain³. A threefold average increase was evident in the morphine-injected group as related to the activity evident in eight other naive mice injected with saline (Table 1; $t=16.8$, $P<0.001$). To test the effects of past experience on the action of morphine another group of mice was placed for 25 min in the toggle-floor boxes and immediately after the end of this session the mice were

Table 1 Effects of morphine on the locomotor activity of naive and experienced mice

Treatment and activity counts		±s.e. during 1st and 2nd sessions	
Group		1st session	2nd session
1	Saline	59.1 ± 3.6	—
2	Morphine	161.4 ± 9.1	—
3	Saline	51.8 ± 3.0	Saline 41.5 ± 2.9
4	Saline	56.5 ± 4.0	Morphine 45.3 ± 3.5

Each session was 25 min long. Groups 1 to 4 were injected with saline or morphine (20 mg kg⁻¹) immediately before the first session. Immediately after the end of the first session groups 3 and 4 were injected again with saline or morphine and tested again. Each group consisted of eight mice.

injected with morphine and tested again for 25 min. The performance during the morphine session was compared with that of a control group subjected to a similar schedule and injected with saline. Table 1 shows that past experience modifies the effects of morphine since no stimulating effect was evident in these conditions in relation to the control group ($t=0.36$, $P>0.05$).

Next we designed another schedule of experience in which the animals were placed for 25 min in the toggle-floor box in the absence of drug. The mice were subjected to a second 25 min test 6 h later and at the end of this session they were injected with saline or morphine and immediately retested during a third 25 min session. By using this procedure the animals were more 'experienced' than in the previous schedule and the injection of morphine not only did not produce a stimulating effect but also resulted in a complete block of their activity (Fig. 1, groups A and B). Visual observation of the behaviour of the mice did not reveal any type of circling or other stereotyped behaviour.

At the end of this session the animals were removed from the toggle-floor boxes and returned to their home pens in which they were caged in groups of eight. When returned to their home cages the morphine-injected mice became very excited and exhibited immediately a very high level of locomotor behaviour. After a few minutes of exploration of their home cage they were returned to the toggle-floor boxes where they exhibited extremely high levels of locomotor behaviour during a 25 min session (Fig. 1, groups A and B, session 4). Thus, exploration of a different environment was followed by the 'running fit' in the same 'experienced' animals in which the injection of morphine previously resulted in a block of their activity. Being in a group of eight (instead of being isolated in the toggle-floor boxes) might have been another reason that contri-

buted to the arousal of the mice. This result was replicated in two additional experiments.

It was possible to induce the 'running fit' in the experienced animals in which morphine resulted in a block of their locomotor activity by leaving the mice in the toggle-floor boxes and switching off the 40 W lamp which was normally illuminating the sound-insulated cabin 1 m above the eight toggle-floor boxes (Fig. 1, group C). Similarly, in another group of animals the action of morphine turned from a blocking to a stimulating effect if a solution with a strong smell of eugenol was sprayed inside the cabin at the end of the morphine session. Thus, experience of the

environment results in a suppression of the morphine-induced running fit or even in a complete block of the animal's activity while a change of the environmental conditions (such as returning the mice to their home cage, a change from light to darkness, or an olfactory stimulus) is followed by the appearance of the 'running fit' effect.

To show that experience of the environment results in a clear change of the behavioural effects of morphine we subjected a group of mice to functional decortication¹¹ while they were subjected to two 25 min sessions in the toggle-floor boxes. In these mice the injection of morphine after the second session resulted in a clear excitatory effect (Fig. 1, group D). Also this finding shows the importance that sensory inputs and experience exert in modulating the effects of the opiate.

While further experiments are necessary to assess why the effects of morphine not only are abolished but result in a suppression of exploratory behaviour in the experienced mice, our findings indicate the importance of the environment in influencing the action of centrally acting drugs¹². Study of these factors in animals might provide a useful tool for analysing the type of effects of morphine and of a number of 'social drugs' in man depending on his social environment.

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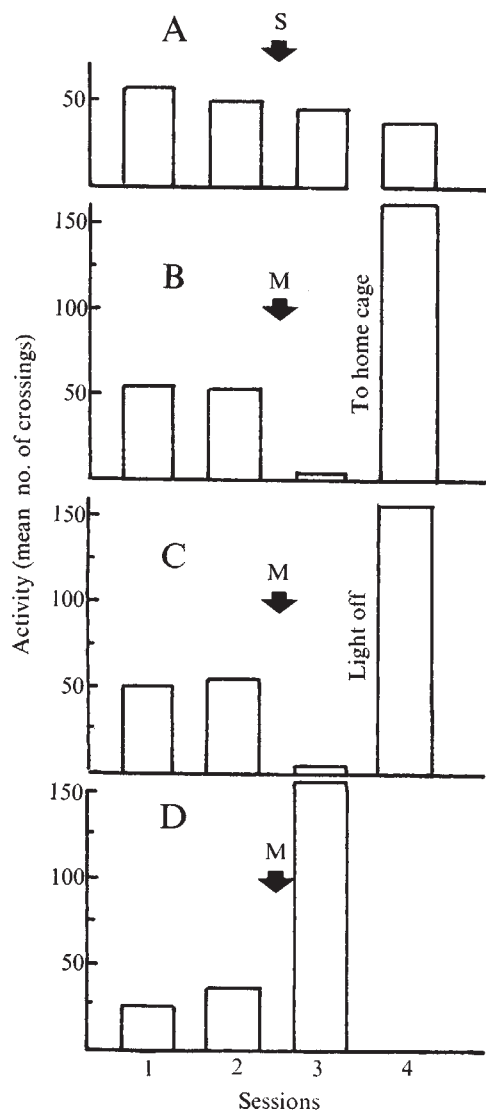


Fig. 1 Effect of past experience on the morphine-induced 'running fit'. Groups A to D were subjected to two 25 min sessions (1 and 2) spaced 6 h apart. Animals of group D were subjected to cortical spreading depression during these two sessions. Immediately after session 2 the mice were injected with saline (S) or morphine (M) (20 mg kg⁻¹) and tested again for 25 min (session 3). At the end of this session the mice were either returned to their home cage for 2-3 min and then tested again for 25 min (session 4) or they were subjected to an additional session in total darkness. The performance of groups A and B during the third and fourth sessions was significantly different ($t=13.1$, $P<0.001$; $t=17.0$, $P<0.001$). Similarly, the performance of group C during session 4 was different from that of a control group (not shown) injected with saline and subjected to a similar schedule ($t=14.9$, $P<0.001$). In the mice tested under spreading depression during sessions 1 and 2 morphine significantly enhanced the performance in relation to that of another group of animals injected with saline ($t=17.9$, $P<0.001$).

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Cervical somato-sensory evoked responses in man

It has been known for over 20 yr that cerebral responses evoked by peripheral nerve stimulation can be recorded from the scalp in man and that they may be altered by disease. But clinical application has been impeded by the variability of the cortical response, particularly of its later components, and by lack of knowledge of its origins. We have explored the possibility of recording from lower levels of the central sensory pathway.

Here we report a response which can be recorded over the back of the neck and base of the skull, which appears to be of nervous origin and has stable properties. It is likely to be useful in the study of neurological disease, notably in conditions such as multiple sclerosis where central conduction may be interrupted or delayed.

Somato-sensory evoked responses (SER) have been recorded

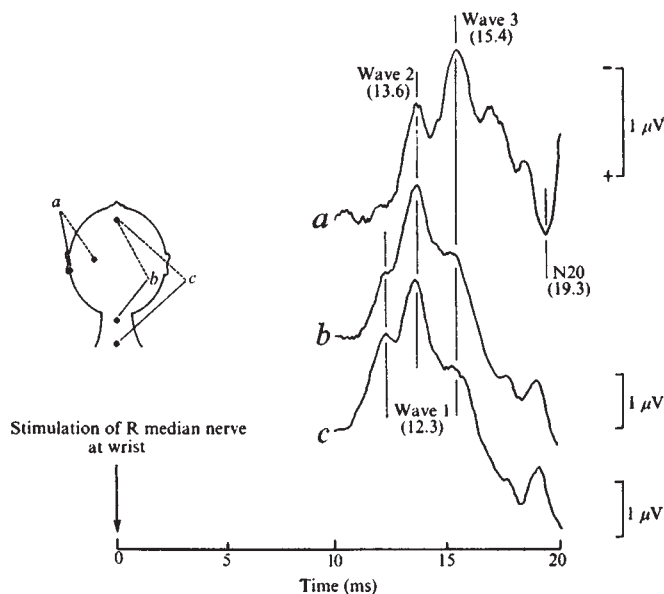


Fig. 1 Cervical somato-sensory responses evoked from a normal 20 yr old female and averaged from 256 stimuli. Electrode placement for ear lobe-parietal region (a) selectively emphasises wave 3. b and c are responses recorded from electrode sites used routinely (C2 and C7).

from surface electrodes over the cervical spinal cord in 30 normal subjects. The stimulus was a square-wave pulse of 0.3 ms duration applied to the median nerve at the wrist. Conventional surface recording and signal averaging techniques were used. Silver disk electrodes were placed between the sixth and seventh cervical spines (C7) and immediately rostral to the second cervical spine (C2) with a common mid-frontal reference electrode (Fz, 10–20 system). The cortical response from the hand area of the contralateral parietal region was simultaneously recorded. The amplifiers had a flat frequency response from 2 to 1,000 Hz. The overall response of the system was 3 dB down at 1,500 Hz, rolling off at 24 dB octave⁻¹ thereafter. The averager sampling rate was 10 points ms⁻¹ and 256 responses were summed.

Preliminary experiments showed that with increasing stimulus strength there was an initial steep rise in amplitude of response and a moderate reduction in latency, both measurements levelling off at stimulus currents approximately three times sensory threshold. A stimulus of approximately four times sensory threshold was therefore used in routine recordings. This produced a tolerable muscle twitch and was not painful. The responses from finger stimulation did not differ from those evoked from the wrist apart from the expected increase in latency and reduction in amplitude. Variation in stimulus rate between 1–10 s⁻¹ produced only minor alterations in the shape of the cervical response, in contrast to the marked changes observed in the cortical response.

With standardised stimulation at a rate of 1 s⁻¹ a predominantly negative wave of complex shape was recorded from all subjects (Fig. 1b and c). The mean amplitude (onset to peak) was 3.0 μV (range 1.4–4.9). The onset latency ranged from 9.4–11.8 ms and peak latency from 11.9–14.3 ms, both measurements being strongly correlated with arm length (Fig. 2). The mean duration was 7.0 ms (range 5.7–8.3). The cervical response was almost completed before the onset of the first component of the cortical SER at about 16 ms. Although there was some variation between individuals, three distinct peaks could be recognised within the cervical SER in all subjects. The major peak (wave 2) was preceded by about 1.3 ms by a minor peak (wave 1) and followed by a further minor peak (wave 3) after a similar interval.

This response is unlikely to be of muscular origin. In relaxed subjects its latency, amplitude and shape were remarkably constant when recorded on different occasions and changes in latency and amplitude with changing stimulus intensity closely paralleled those of the first major component of the cortical SER (N20–P28), which is accepted as neurogenic. Activity in the neck muscles due to anxiety or voluntary contraction obliterated the response whereas a myogenic response would have been enhanced. Goff, *et al.*¹ recently recorded, but did not characterise, a wave of similar latency (N14) over the cervical spine evoked by stimulation at the wrist and presented convincing evidence that it was neurogenic.

Cracco² recorded from surface electrodes at various sites over the spinal cord following peripheral nerve stimulation and concluded from changes in the onset latency that the evoked response was a travelling wave. We found no such increase in latency between the lower and upper neck. The peak latencies of all three components were identical when recorded from caudal and rostral sites over the cervical spinal cord and there was no consistent change in the less sharply defined onset latency. The relative amplitudes of the three components showed systematic variation with recording site, that of wave 1 being always greatest at C7, falling off both rostrally and caudally. The amplitude of the main peak (wave 2) was similar when recorded at C7 and C2. Wave 3 could be emphasised selectively by recording from mastoid or earlobe. Some of these variations are illustrated in Fig. 1. The findings with regard to both latency and amplitude are more consistent with generation of the components of the cervical response from fixed sites than with a travelling wave in the dorsal column system. The possible locations of these sites, which may include dorsal root ganglia, spinal cord interneurons, dorsal column nuclei and cerebellum, have not yet been elucidated. There appears to have been no relevant comparison of surface with direct recordings in the experimental animal.

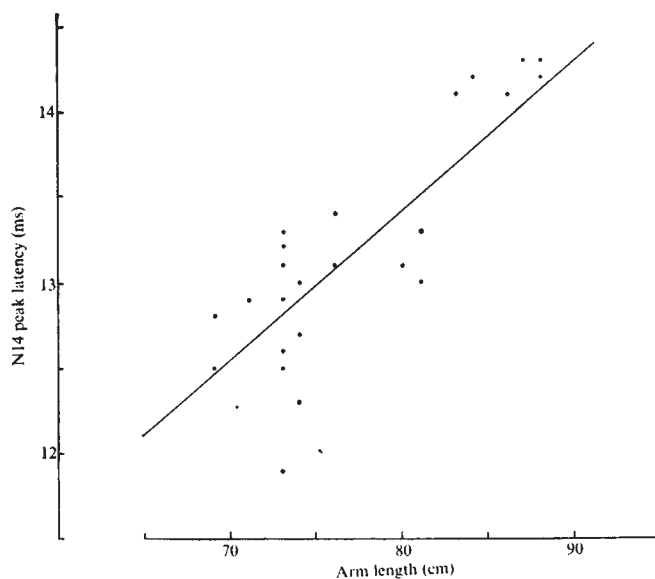


Fig. 2 Relationship between peak latency of cervical response evoked by median nerve stimulation at wrist and arm length measured from wrist to seventh cervical spine (correlation coefficient 0.85).

Surface recordings, in contrast to recordings from the epidural space³, are readily repeatable in normal subjects and patients with neurological disease. Work is now in progress designed to clarify the sites of origin of the components of the cervical SER and their use in the study of disease of the central nervous system.

This work was supported in part by the Multiple Sclerosis Society of Great Britain.

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Received July 29, 1974.

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Luteinising hormone and testosterone in man

In mammals, including man, specific sexual stimuli can affect the secretion of luteinising hormone (LH) from the pituitary and testosterone from the testis. For example, the presence of the female and copulation is associated with an increase in the blood levels of testosterone in rabbit¹, rat², bull³ and man⁴, and in each species excluding man this has been shown to be related to an elevation of blood plasma LH^{1,3,5}. Among animals, merely the presence of a receptive female may induce these changes in the absence of intercourse^{2,3,5} indicating that in some species visual and olfactory cues alone are sufficient to elicit an endocrine response. In man the anticipation of female company after long periods of abstinence apparently has similar effects⁶. Very few studies of this sort have looked at both LH and testosterone secretion simultaneously in sufficient detail to provide convincing evidence of short term endocrine changes related to behaviour. The aim of my study was to establish whether the normal pulsatile secretion of LH and testosterone in men could be altered by a specific sexual stimulus. It was decided to expose male volunteers to a film sequence which included scenes of human behaviour which were judged to be sexually provocative.

Eight healthy men aged between 24 and 29 yr were recruited. Blood samples were collected at intervals of 10–40 min using an indwelling needle inserted into a forearm vein. The trials lasted 6–7 h starting at about 1700h and only two or three subjects were studied in any one evening.

After a meal each subject spent the evening relaxing, reading and talking. The film projector was placed in a separate room and each person could view the film in complete isolation, and was free to study and relate to the scenes as he wished. None of the volunteers knew at what time they would be called to the room; they were informed at the beginning of the trial that the film may or may not contain scenes of sexual interest, and later they each completed a questionnaire indicating their reactions during the evening.

Seven out of eight subjects recorded the film sequences as sexually stimulating to a greater or lesser degree, and experienced full or partial erection of the penis at this time. The feeling of sexual excitement persisted in these subjects at the end of the viewing period.

There were no changes in blood levels of LH or testosterone in the subjects which could be definitely related to the films (Fig. 1). Plasma LH concentrations changed markedly during each trial period; intermittent high values were followed in many cases by progressively decreasing values producing a pattern indicative of the documented pulsatile release of gonadotrophins from the pituitary^{7,8}. These discharges occurred at irregular intervals; two out of eight subjects produced an LH surge during the viewing period which had a frequency not greater than expected in an unstimulated situation (LH surge in a resting situation

every 2–4 h would produce one or two releases in any 30 min period in eight subjects). One noticeable feature was that LH values were generally high in all subjects at the beginning of the trial, which seemed to be related to the experience of the initial vein puncture.

Testosterone concentrations fluctuated during the evening in a normal pattern^{9–10} unrelated to the film period. By relating the testosterone values to the main LH peak for each individual and calculating a mean steroid level for the eight studies, it was possible to demonstrate a temporal relationship between LH and testosterone concentrations in the serial samples (Fig. 2). An endogenous surge in LH was associated with a progressive rise in testosterone levels which were at a maximum (+26% of value at LH surge) at 90 min after the increased LH stimulus. There was considerable variation, however, in the timing and extent of the testis response in the different cases.

I conclude that normal male subjects can experience varying degrees of sexual excitement without any concomitant change in blood LH and testosterone levels. Very few studies on man have revealed short term endocrine changes related to behaviour. Fox *et al.*⁴, studying a single individual claimed to have found significantly elevated plasma testosterone levels during and within 5 min of coitus, and similar findings are indicated by Steinbeck¹¹. Yet there is no evidence that these steroid changes are induced by elevated gonadotrophin levels^{4,12}. Indeed, the present study illustrates that the human testis is relatively sluggish in its response to endogenous changes in LH, and it can be assumed that any sexual stimulus affecting LH would not

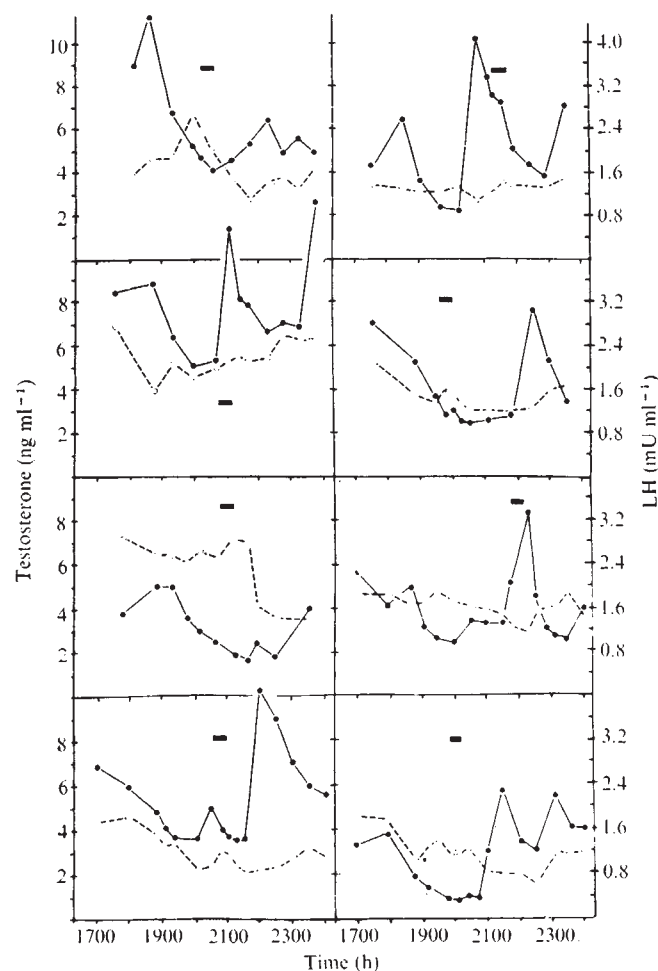


Fig. 1 Blood plasma levels of LH (●) and testosterone (○) measured by radioimmunoassay^{10,18} in eight normal men for a 7 h evening period during which they viewed a short sexually stimulating film. —, Film.

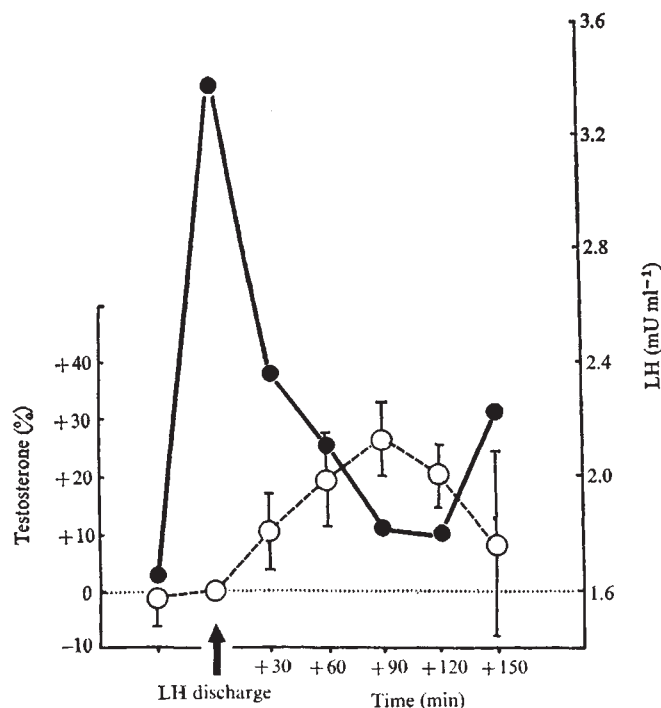


Fig. 2 Changes in the plasma concentration of testosterone in the eight subjects (mean $\pm$ s.e.) related to the principle LH peak for the individual studies. A temporal relationship between LH (●) and testosterone (○) is apparent, with testosterone values increasing after the LH discharge and reaching a maximum 90 min later.

show itself in terms of changes in plasma testosterone for some 30–90 min. It seems probable that the observations of Fox and Steinbeck may be accounted for in terms of increased blood flow through the testis which is likely to be important in causing a transitory increase in testosterone¹³. Neural pathways involving the hypothalamus need not be implicated in this response.

While no report has yet described short term changes in LH secretion in man related to sexual stimuli, there still remains the observation that LH levels in my study were increased at the beginning of each trial period. This involved the very first blood sample in several cases and it seems likely that the experience of having the blood sampling needle fitted into the arm may have caused this response; the LH surge occurred at the time of the venapuncture or during the hour afterwards. Studies on 'stress' associated with surgery¹ and army training¹⁵, as well as foot shock in rats¹⁶ and fighting in monkeys¹⁷ lead one to expect decreased LH secretion under these conditions. The observation remains, however, as a cautionary note to those using venapuncture to study gonadotrophin levels and it seems possible that a single skin puncture has a greater impact on the hypothalamic/pituitary axis than a 30 min sexually stimulating film.

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Received July 1, 1974.

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Muscular dystrophy and muscle cell death in normal foetal development

ALTHOUGH the application of modern biochemical, morphological and experimental techniques to the study of muscle disease has greatly extended our knowledge of myopathic processes, the cause of muscular dystrophy continues to elude investigators. McComas *et al.*¹ have suggested that the motor neurones rather than the skeletal muscle fibres are primarily affected but this has been seriously questioned^{2,3}. My own hypothesis is based on the concept of embryonic cell death and stems from observations that I, as well as others, have made on the death of muscle cells in normal human foetuses^{4,5}.

Cell death in embryogenesis takes place in a highly predictable manner and all the evidence points to it being genetically controlled⁶. It is a familiar process in eliminating tissues and organs useful only during embryonic life, in the elimination of phylogenetic vestiges and in modelling processes such as the formation of clefts or digits. The death of embryonic cells in a tissue such as muscle, where the elimination of cells appears, on the face of it, to have no useful purpose, must nevertheless serve a function which is probably crucial to the subsequent healthy development and growth of that tissue.

I wish to suggest that the basic defect in muscular dystrophy is due to a derangement in the normal process of muscle cell death during a critical stage in development—possibly between weeks 10 and 16 of foetal life. From previous observations I have made in human foetuses, muscle cell death can be seen up to about week 16: this is roughly in agreement with Boyd's observations^{4,5}.

The derangement in the cell death process might come about in one of three ways. First, the cell death 'switch' may simply not be activated at the critical stage in muscle development. As a result the developing muscle fibres do not go through the proper embryological processes (of which cell death is a part) and which are essential for the healthy development of the tissue. The imperfectly formed muscle fibres would then die off prematurely. Second, the cell death switch may not be activated at the correct time in the lifespan of the individual. Instead of occurring between weeks 10 and 16 of foetal life it is delayed until later in development, or even into the post-natal period. On this basis muscular dystrophy can be looked upon as a normal process but one which has occurred too late to perform its role in development. Third, the cell death mechanism may be triggered off at the correct time but the failure is one where the process cannot be turned off—the muscle cell death would continue in a relentless fashion until

virtually all the muscle fibres had died. Here again muscular dystrophy may be thought of as a normal process but one which continues beyond the period when it is able to play any useful role. One way in which this could come about would be if the target cell (muscle fibre) contained a blocking mechanism which in some way failed to neutralise the effects of the agent responsible for the cell death.

Relatively little is known about the way in which cells die during embryogenesis but it is known that hormones (for example oestrogens, androgens or thyroxine) are involved in certain specific instances. It has been shown that in amphibia thyroxine can break down the gills, tail and larval head muscles, whilst in organ cultures of tails of *Xenopus* the addition of appropriate concentrations of thyroxine causes regression of the tissues⁶. Other possible mechanisms of cell death include the senescence of cells in which excessive amounts of membranous material accumulate until the point is reached when the metabolism of the cell is impaired. Errors in the metabolism of proteins which build up in the cytoplasm and block further cell respiration is another possibility⁷. In the case of embryonic cell death the elimination of cells is an extremely rapid one and it is difficult to accept that cell senescence or a build up in abnormal proteins could act sufficiently quickly. On the other hand a genetic mutation or the production of metabolite which specifically blocks cell respiration could cause very rapid death of cells. Such a specific blocking metabolite might act in a somewhat analogous fashion to the injection of certain fluoro compounds into laboratory animals, which causes very rapid impairment of cell function and structure by blocking the oxidative metabolism of cells in a highly specific manner⁸.

Morphological studies provide no clue as to the mechanism of cell death, but in the regression of tails of *Xenopus* larvae the death of muscle cells is accompanied by phagocytosis and much lysosomal enzyme activity⁹. Although in humans the

degenerating foetal muscle may be surrounded by mononuclear mesenchymal cells, phagocytosis does not seem to take place on any significant scale⁴. In a recently examined 12-week-old foetus, however, I have observed the apparent ingestion of degenerate cell products in skeletal muscle (Fig. 1).

If, as I am suggesting, the muscular dystrophies are primarily disorders of muscle fibres, why, it may be asked, are some muscle groups severely affected while others are spared, or at any rate affected to a much lesser extent? My hypothesis goes some way to explaining this in that there is evidence to suggest that cell death is extensive in some muscle groups but is less marked or hardly occurs at all in others⁵.

In my view, to discover insights into the cause of muscular dystrophy, we should look to normal developmental processes in human foetal muscle and in particular the phenomenon of muscle cell death. I have suggested ways in which an upset in this phenomenon might lead to muscular dystrophy, so that these diseases can be looked upon as a normal process but one occurring at the wrong time in the individual's life span or else one which has not been repressed. Further studies of muscle cell death in normal fetuses and the fetuses of mothers who are carriers of the dystrophy gene are needed.

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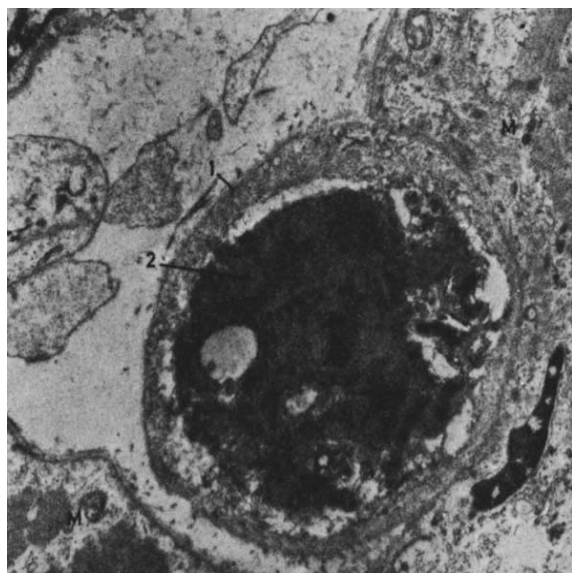


Fig. 1 Foetal muscle at 12 weeks. Quadriceps muscle was obtained from apparently normal human fetuses of either sex fresh from therapeutic abortions. Cubes of tissue (1 mm³) were fixed in 3% buffered glutaraldehyde at 4°C (pH 7.3) for 4 h. This was followed by washing in buffered sucrose, post-fixation in OsO₄, dehydration and embedding in araldite. Thin sections were stained on copper grids with uranyl acetate and lead citrate. The tissue was examined with an AEI EM6B electron microscope. To eliminate the possibility of traumatic artefact in four cases an entire lower limb was fixed immediately in 4% formaldehyde in buffered saline at 4°C and left for 24 h. Small pieces of quadriceps were then post-fixed in 2% OsO₄ and processed as before. (1) Phagocytic cell containing ingested cell products (2), with identifiable nuclear remnant (3), M line myotubes. (×10,000).

Liver as major organ of phenol detoxication?

THE ability to detoxicate and eliminate potentially harmful substances from the body represents a defence mechanism, the importance of which is well established. It has been assumed that the protective devices themselves, the detoxication mechanisms, have evolved to protect the organism from potentially harmful chemicals occurring naturally in the environment. These chemicals, usually described as foreign, are rendered harmless by metabolic transformations and modified compounds are subsequently excreted usually in the urine or bile. The liver is generally regarded as the major organ of detoxication.

The work we report here, on the fate of phenol in the rat, necessitates a reappraisal of some hitherto accepted aspects of detoxication. In particular, it focuses attention on the need to re-assess the role of the liver in detoxication.

Whole-body autoradiograms¹ were prepared using young rats (approximately 50 g body weight) which had received U¹⁴C-phenol (10 μCi; 20–40 mCi mmol⁻¹) either orally or intraperitoneally. The autoradiograms obtained when U¹⁴C-phenol was administered by either route showed that the level of isotope in areas corresponding to the liver did not, at any time, exceed that of the blood. Thus, the liver has no ability to concentrate either phenol or its metabolites suggesting that U¹⁴C-phenol either fails to enter the liver cells or alternatively, that the radioactive molecules have a transient cellular existence due to rapid turnover in the liver. The qualitative

patterns do suggest, however, that the liver is not the principal site of phenol detoxication. Experiments were therefore designed to investigate further the contribution of the liver and determine the sites and extent of extrahepatic detoxication. As phenolic substances are normally presented to the animal through the gastrointestinal tract, initial studies were made to determine the detoxication capacity of the gastrointestinal tract with respect to phenol.

Isolated rat gut preparations were perfused² with Krebs-Henseleit buffer pH 7.0 containing glucose (500 mg per 100 ml). Segments (30–50 cm) of small intestine were washed free from debris by perfusing with 50 ml of the perfusion medium before attaching to the apparatus. U¹⁴C-Phenol was added to the mucosal medium and samples were withdrawn from the circulating serosal and mucosal media over 2 h. Analysis of these samples showed a decrease in the level of radioactivity in the mucosal circulation and a concomitant increase in the level of radioactivity in the serosal circulation. When either U¹⁴C-phenol (10 μ Ci) or 10 mg of phenol mixed with U¹⁴C-phenol (10 μ Ci) was added to the mucosal medium 50% and 78% respectively of the radioactivity was transferred to the serosal circulation in 2 h. At the end of the experiments the radioactivity in both media was distributed between phenyl sulphate (5%) and phenyl glucuronide (95%). No unchanged U¹⁴C-phenol could be detected.

Conjugation of phenol by gut preparations *in vitro* raises the possibility that the gut is the principal site of detoxication of phenol ingested in the diet and that phenol is transported into the blood after conjugation. This was explored in the whole animal by perfusing the small intestine *in situ* with media containing U¹⁴C-phenol and simultaneously sampling blood directly from the hepatic portal vein.

Wholly anaesthetised rats (phenobarbitone) with bile-duct catheters were prepared and the gut was ligated both at the duodenal-jejunal flexure and at a point about 30 cm distal to the flexure. After washing, to remove intestinal debris, the isolated gut segment was perfused (3 ml min⁻¹) *in situ* with water (30 ml) and the perfusate returned to a mixing reservoir for recirculation. Bile was allowed to join the perfusate circulation at the mixing reservoir.

U¹⁴C-Phenol (10 μ Ci) or 10 mg phenol mixed with U¹⁴C-phenol (10 μ Ci) was added to the circulating perfusate and samples (1 ml) were withdrawn at 30 min intervals over 3 h. Portal blood samples (0.5 ml) were collected through the pancreaticoduodenal vein at 30 min intervals into heparinised tubes and the plasma was removed after centrifuging. In both experiments there was a progressive decrease in the amount of radioactivity in the circulating perfusate. Over the 3 h experimental period the major proportion of the radioactivity was transported from the intestinal lumen and was accompanied by the appearance of radioactivity in portal blood. In all plasma samples, conjugates of U¹⁴C-phenol only were found; U¹⁴C-phenol itself could not be detected. Phenol conjugates appeared in the intestinal perfusate within 30 min but at this time the majority of the radioactivity was still present as unchanged U¹⁴C-phenol. In contrast, after 2 h perfusion, unchanged U¹⁴C-phenol could not be detected and the radioactivity was present as phenyl sulphate (72%) and phenyl glucuronide (28%).

These results strongly support the view that free phenol is not transported as such from the intestinal lumen but in conjugated form. It therefore follows that the role of the liver is minimal in the detoxication of orally ingested phenol. When the gastrointestinal tract is by-passed by intravenous administration of free phenol, however, conjugation with sulphate and glucuronide and subsequent excretion of these conjugates in the urine nevertheless takes place³. In these conditions the liver may assume major importance as a centre of detoxication. This possibility was explored in experiments in which phenol was administered to hepatectomised rats.

Rats were anaesthetised with phenobarbitone and the ureters were cannulated. In test (hepatectomised) rats, hepatic

tissue (60%), the spleen and the gut were removed following appropriate ligations. When U¹⁴C-phenol was administered intravenously to hepatectomised and control rats at dose levels of 5 mg kg⁻¹ and 10 mg kg⁻¹ body weight the percentage of radioactivity recovered in urine of test and control animals over 3 h was essentially the same. Furthermore, test and control urines contained the conjugates of phenol although the glucuronic acid conjugate was proportionally higher in the test than in control urines. Thus, even in the absence of the liver and gastrointestinal tract, formation and urinary secretion of phenol detoxication products still occurs.

Collectively, the results strongly suggest that the detoxication aspect of liver function has been over-emphasised for it is shown that the liver is not essential to the detoxication of phenol. Attention is drawn to the considerable capacity of the gastrointestinal tract to detoxicate phenol. The findings of this investigation point to a major role for the gastrointestinal tract in the protection of the whole organism from the potential toxic effects of phenol. The necessity for such strategically-placed detoxication devices is immediately apparent when it is recalled that up to 600 mg of phenolic material may be ingested each day in the normal human diet. The experiments reported here, demonstrate that, in the case of phenol, this requirement is satisfied by the intestinal barrier. Other potential detoxication sites are thus not presented with exogenous phenol since it enters the portal blood in conjugated form only. The importance of this latter observation is emphasised by the known effects of phenol on the red blood cells and, in retrospect, it is clear that the transport of phenol in unconjugated form by the blood represents a highly undesirable and potentially hazardous process with respect to the health of the whole organism.

J.J.M. is grateful to the Science Research Council for a CASE Fellowship, supported by May and Baker, Ltd, Dagenham, Essex, UK. We thank the Wellcome Trust for financial support.

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Cardiotoxic protein from edible mushrooms

THE cardiotoxic protein volvatxin has been isolated from the edible mushroom, *Volvariella volvacea*¹. We have now isolated another cardiotoxic protein from the edible mushroom *Flammulina velutipes* (Curt. ex Fries) Sing., which is widely eaten in the Orient and is canned for local consumption and export. We have called this protein flammutoxin.

Flammulina velutipes fruits throughout the year. Its cap, initially ovoid and yellow becomes, within one or two days, flat and yellowish brown with an edge of lighter colour. The cap reaches a diameter of 2 cm; the stem achieves a length of 15 cm and a diameter of 0.5 cm. This mushroom has little taste but a slightly unpleasant smell.

The method of isolation of flammutoxin was similar to that of volvatxin¹ except that flammutoxin was obtained between 0 and 60% saturation of ammonium sulphate. The

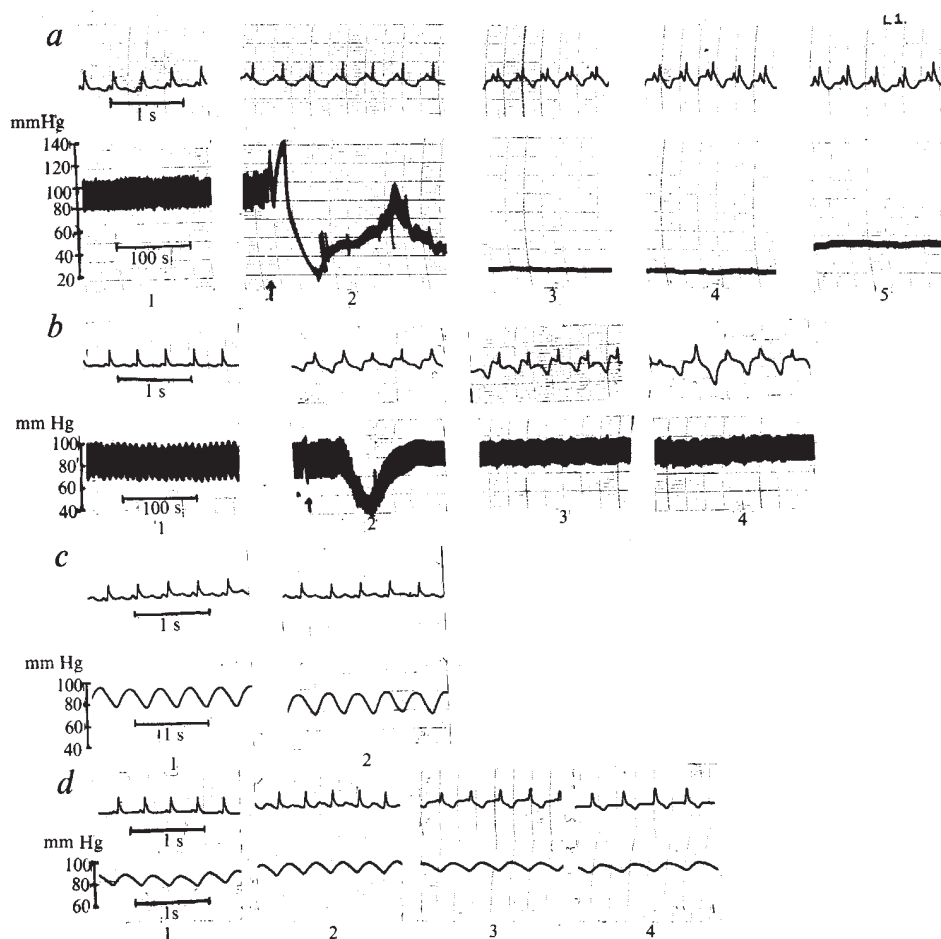


Fig. 1 Effect of *a*, flammutoxin and *b-d*, volvatoxin on the electrocardiogram and arterial blood pressure of a 2 kg cat, pentobarbital anaesthetised (30 mg per kg body weight intraperitoneally). Upper tracing ECG (lead II); lower tracing, arterial blood pressure. *a*, 0.25 mg per kg body weight flammutoxin was injected. 1, 0 time; 2, 1 min; 3, 10 min; 4, 25 min; 5, 60 min; Arrow indicates the time of injection. *b*, Injection of 1 mg per kg body weight volvatoxin A₂; 1, 0 time; 2, 1 min; 3, 10 min; 4, 60 min. *c*, Injection of 5 mg per kg body weight volvatoxin A₁; 1, 0 time; 2, 60 min. *d*, Injection of 2 mg per kg body weight the mixed solution of volvatoxin A₁ and A₂ (weight ratio 1:2). 1, 0 time; 2, 3 min; 3, 5 min; 4, 10 min.

concentration of flammutoxin in the mushroom was 320 mg kg⁻¹ whereas that of volvatoxin A was 1,100 mg kg⁻¹. The toxicity of flammutoxin when administered intraperitoneally was determined as described previously¹. The LD₅₀ was found to be 2.45 mg per kg body weight. The toxicity can be completely eliminated by heating a solution of flammutoxin at 100°C for 20 min. Flammutoxin has three biological activities similar to those volvatoxin: (1) direct haemolytic action against human group O blood cells at a concentration of 25 µg ml⁻¹; (2) ability to cause a writhing reaction with a delay before onset at a dose of 1.2–2 mg kg⁻¹ body weight and twenty-two writhing reactions within 10 min of the first writhing reaction;

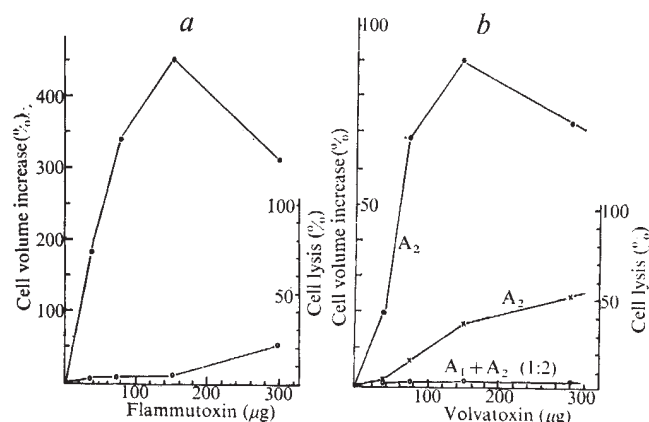


Fig. 2 Effect of *a*, flammutoxin and *b*, volvatoxin on the swelling of Ehrlich ascites tumour cells. The tumour cells (2×10^8 ml⁻¹) were treated with various concentrations of flammutoxin. The reaction mixture was incubated at 37°C for 5 min and it was centrifuged at 2,300g for 30 min at 4°C with a Wintrobe tube³. ●, Cell volume; ○, cell lysis.

Table 1 Amino acid composition of flammutoxin

Amino acid	Residues per 10,813
Lysine	7.84 (8)
Histidine	0.64 (1)
Arginine	2.10 (2)
*Tryptophan	5.80 (6)
Aspartic acid	8.93 (9)
Threonine	11.82 (12)
Serine	9.72 (10)
Glutamic acid	8.00 (8)
Proline	4.10 (4)
Glycine	8.15 (8)
Alanine	4.02 (4)
Valine	6.88 (7)
Isoleucine	4.02 (4)
Leucine	5.72 (6)
Tyrosine	3.88 (4)
Phenylalanine	3.10 (3)
Total	96

* Measured by alkaline hydrolysis method⁶.

(3) effect on the ECG at a dose of 0.25 mg per kg body weight, causing depression of the ST segment and inversion of the T wave (Fig. 1a).

Flammutoxin also has several activities which were not found in volvatoxin A. First, flammutoxin induces a sharp fall in blood pressure (Fig. 1a). Second, it causes the swelling of Ehrlich ascites tumour cells (Fig. 2a). Third, it inhibits the respiration of Ehrlich ascites tumour cells (Fig. 3).

The probability that flammutoxin, like volvatoxin, might consist of two components was tested by gel filtration² and SDS acrylamide gel electrophoresis³. Flammutoxin was found to contain only one type of molecule with a molecular weight of 22,000. Table 1 shows its amino acid composition⁴ which is similar to that of volvatoxin A₂. The molecule is rich in lysine;

its isoelectric point is 4.2. The two components of volvatoxin A, A1 and A2, were tested to see whether either of them separately possessed those properties found in flammutoxin but not in volvatoxin A. Volvatoxin A2 was found to cause a decrease of blood pressure (Fig. 1b), lysis of human group O red blood cells, swelling of tumour cells (Fig. 2b), as well as the inhibition of respiration (Fig. 3) of Ehrlich ascites tumour cells. Volvatoxin A2 alone cannot however elicit the writhing reaction.

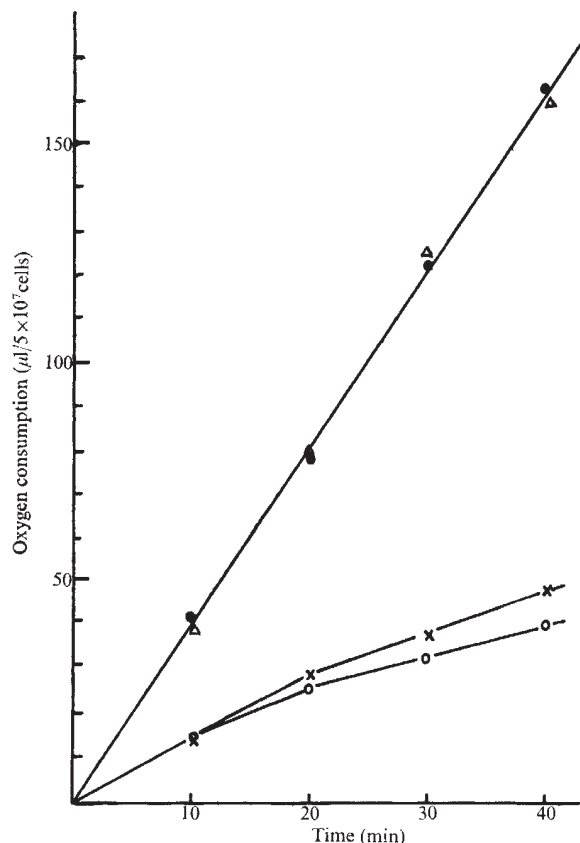


Fig. 3 Effect of flammutoxin on the respiration of Ehrlich ascites tumour cells. One millilitre of Ehrlich ascites tumour cells ($5 \times 10^7 \text{ ml}^{-1}$) was incubated with flammutoxin and the oxygen consumption was measured by manometric method with a Warburg apparatus (B. Braun Apparatebau, Melsungen, West Germany). ●, Control; ×, volvatoxin A2 ($50 \mu\text{g ml}^{-1}$); ○, flammutoxin ($20 \mu\text{g ml}^{-1}$); Δ, volvatoxin A1 ($20 \mu\text{g ml}^{-1}$) plus volvatoxin A2 ($50 \mu\text{g ml}^{-1}$).

The activities of volvatoxin A2 were completely abolished by the presence of volvatoxin A1 at weight ratios of volvatoxin A1 to A2 higher than 1:2 (Figs 1a, 2b, 3). Volvatoxin A1 has none of biological activities shown either by volvatoxin A or volvatoxin A2. We tested whether volvatoxin A1 could affect the biological activities of flammutoxin as it affected volvatoxin A2, but we found that it had no effect on flammutoxin.

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Dopamine 3-O-sulphate, an end product of L-dopa metabolism in Parkinson patients

PREVIOUS studies from these laboratories¹ have shown that L-3,4-dihydroxyphenylalanine (L-dopa) does not undergo sulphonconjugation *in vitro* in the cytosol sulphating system of rat tissues whereas dopamine very readily yields a mixture of the 3- and 4-O-sulphates. These observations might therefore serve to explain earlier claims² that sulphonconjugated dopamine constituted a significant proportion of the amine fraction extractable from rat tissues following the administration of ¹⁴C-labelled L-dopa and raises the question of the role of sulphonconjugation in dopamine metabolism. In man this may be of significance in L-dopa therapy in the treatment of Parkinson's disease since such conjugates might represent either intermediates or end products of metabolism.

The administration of ¹⁴C-labelled L-dopa to normal people is followed by the excretion of sulphonconjugates of dopamine in the urine³ and Parkinson patients on high oral doses of L-dopa have been shown to excrete up to 80% of the amine in this form⁴⁻⁶. It therefore seems that sulphonconjugation plays a significant role in the elimination of dopamine from the system.

In all of these studies, however, no definite proof of the absolute identity of the excretion products has been obtained. It has generally been concluded that the 4-O-sulphonconjugate is excreted but in view of our earlier findings that *in vitro* in rat liver preparations, the 3-O-sulphate is the principal product of sulphonconjugation, such a conclusion might seem incorrect. Furthermore we have shown (W.N.J., and F.A.R., unpublished) that in the rat the 3-O-sulphate is metabolically inert. It does not undergo desulphation when incubated under a variety of conditions with preparations of the three arylsulphohydrolase enzymes from rat tissues and when administered intraperitoneally or intravenously to rats it is rapidly excreted in the urine unchanged. In these experiments the 4-O-sulphate behaved somewhat similarly although in contrast it underwent appreciable metabolism *in vivo* before excretion. Attempts have therefore been made to determine the identity of dopamine sulphonconjugates excreted by Parkinson patients on high doses of L-dopa. Urine samples (24 h) were collected from patients attending an out-patients clinic and were stored at -20°C over 2 g of sodium metabisulphite as preservative. Before use the thawed urine was filtered through Whatman No. 1 paper to remove any sediment and a 50 ml aliquot of the filtrate was lyophilised after the addition of 20 μg each of authentic dopamine 3-O and 4-O-³⁵S-sulphate (prepared as described previously¹, specific activity $\approx 1 \mu\text{Ci mg}^{-1}$) in 2 ml of water. The addition of isotopically labelled conjugates aided in locating the relative positions of the excreted metabolites and eventually in determining the completeness of their recovery. The freeze-dried sample was then dissolved in 10 ml of water and applied to a column ($1.4 \times 35 \text{ cm}$) of Dowex 1 $\times$ 8 ion exchange resin (200-400 mesh, acetate form) which had been previously well washed with water. The column was eluted with 0.2 M acetic acid at a flow rate of about 0.5 ml min^{-1} and 10 ml fractions collected. The eluate was continuously monitored at 280 nm by means of an L.K.B. Uvicord system and radioactivity in the fractions was detected by counting 1 ml of the samples mixed with 10 ml of scintillator (toluene, 1 l; Triton X 100, 500 ml; 2,5-diphenyloxazole, 5 g) in a Packard Tri-Carb liquid scintillation spectrometer.

The dopamine 3-O-³⁵S-sulphate was eluted between 900 and 1,250 ml and the 4-O-³⁵S-sulphate between 1,300 and 1,700 ml

Table 1 Daily excretion of dopamine sulpoconjugates by Parkinson patients on L-dopa therapy

Subject	Dose of L-dopa (g per 24 h)	Dopamine 3-O-sulphate (mg per 24 h)	Dopamine 4-O-sulphate (mg per 24 h)	Total dopamine sulpoconjugate (mg per 24 h)	Dopamine 3-O-sulphate (% of total)	Dose excreted as dopamine sulpoconjugate (%)
1 male, 54 yr	4	292.9	78.2	371.1	78.9	7.8
2 male, 38 yr	3	245.3	39.5	284.8	86.1	8.0
3 male, 70 yr	3	97.5	16.2	113.7	85.7	3.2
4 male, 52 yr	2	242.1	45.3	287.4	84.2	12.2

(as determined in preliminary experiments). Two peaks of ultraviolet absorption which were clearly separated from the main absorbing band and exactly coincident with the two peaks of radioactivity were observed (Fig. 1). The fractions from the column representing each sulpoconjugate were pooled and the radioactivity of each was measured on 1 ml aliquots to determine the total recovery of the sulpoconjugates from the column. A portion (100 ml) of each sulpoconjugate fraction was lyophilised and a sample (1 ml) of the solution obtained by dissolving the residue in 4 ml of water was subjected to hydrolysis by heating at 100°C with 2 ml of 0.3 M HCl for 40 min. This procedure had been shown in previous experiments to hydrolyse 96.0% of both the 3-O and 4-O-sulphates without destruction of the liberated dopamine which could then be determined. Portions (2 ml) of the hydrolysates were then diluted with water to 250 ml for the 3-O-sulphate and to 25 ml for the 4-O-sulphate fractions. This dilution of the fractions was sufficient to make adjustment of their pH unnecessary. The diluted hydrolysates were then analysed for dopamine using the trihydroxyindole procedure⁷. The purity of the hydrolysates with respect to dopamine was confirmed by an examination of their excitation and emission spectra. The presence of other catecholamine derivatives could not be detected. The results were corrected for the amount of ³⁵S-labelled sulpoconjugate added at the beginning, the recovery (95%) from the ion-exchange columns and also the efficiency of the hydrolysis procedure (Table 1).

The amount of dopamine sulpoconjugate excreted by these Parkinson patients is comparable with that found by other workers although this was not the main concern of the present study. The results however show that the 3-O-sulphate is certainly the predominant conjugate and is consistently so, even in patient no. 3 where the total amount excreted is rather low. It therefore seems that the sulpoconjugation of dopamine in the human follows a pattern similar to that in the rat and can result in the rapid removal of an appreciable amount of

administered dopa from the system. It is also noticeable that the amount of amine sulpoconjugated becomes considerable when dopa is administered orally, emphasising the possible role of the digestive tract in this process. Attention has been drawn to this point in earlier studies on the metabolism of related compounds⁸. In the mammalian system O-sulphation and O-methylation appear to occur predominantly on the same OH group and they may therefore represent competing processes. The predominant position of O-methylation in various catechols is not only dependent upon the relative nucleophilicities of the phenolic groups but is also strongly influenced by the polarity and orientation of the substituent side chain and other factors⁹. In the case of dopamine, 3-O methylation is favoured near physiological pH. Although less is known about the effect of such factors on the O-sulphation of catechol derivatives, it seems that the situation is somewhat similar (unpublished results). The site of formation of excreted dopamine sulpoconjugates in the system now needs to be established. The identity of the dopamine sulpoconjugate to be found in the tissues² and in the blood where it has been claimed to be the only detectable form of the amine¹⁰, should also be investigated.

The method developed for the identification of the metabolites seems to be generally applicable (W.N.J. and F.A.R., unpublished) and can be of use for the analysis of normal human urine where the excretion pattern is similar although the concentrations are appreciably lower¹¹.

We thank Roche Products Ltd for financial support and for arranging access to the patients. W.N.J. is grateful to the SRC for a CAPS studentship.

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Received July 23; revised August 23, 1974.

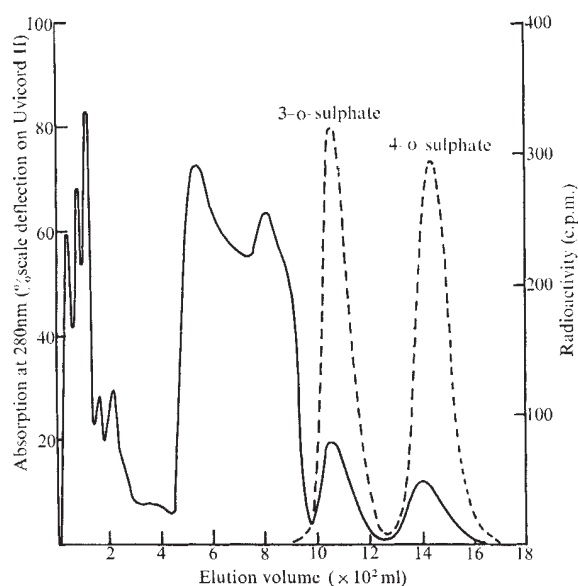


Fig. 1 Elution profile of urinary dopamine sulpoconjugates. —, Absorption at 280 nm; ---, radioactivity.

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Displacement of bound ¹⁴C-fluphenazine by biogenic amines and antipsychotic drugs in homogenates of brain tissue

CONSIDERABLE evidence suggests that antipsychotic drugs block dopamine receptors at postsynaptic sites in the central nervous system^{1,2}. Receptor sites for neurotransmitter sub-

stances, such as acetylcholine³⁻⁵ and glycine⁶, have been studied by measurements of the direct binding of radiolabelled compounds that have high affinity for such sites and cause receptor blockade. In a similar study of biogenic amine receptor sites, I have measured the binding and displacement of the potent phenothiazine antipsychotic drug, fluphenazine, to preparations of brain tissue *in vitro*. I have found that fluphenazine binds to all regions of the rat brain, and that the potency of antipsychotic drugs in displacing fluphenazine correlates well with their clinical efficacy. Dopamine does displace fluphenazine from binding sites, but this is a nonspecific effect shared by other biogenic amines.

¹⁴C-fluphenazine (4-[3-¹⁴C-3-(2-trifluoromethyl) phenothiazin-10-yl] propyl-1-piperazine ethanol, dihydrochloride, 2.4 mCi mol⁻¹) was synthesised by F. Dondzila (Chemical Development Department, Squibb Institute) by first reacting 1-chloropropyl-3-¹⁴C-bromide (3 mCi mmol⁻¹, New England Nuclear Corp.) with 2-(trifluoromethyl) phenothiazine to form 10(1-¹⁴C-3-chloropropyl)-2-(trifluoromethyl) phenothiazine. This compound was refluxed in the presence of 2-piperazine ethanol to form ¹⁴C-fluphenazine base from which the dihydrochloride salt was obtained. ¹⁴C-fluphenazine dihydrochloride was recrystallised twice from absolute ethanol to yield 98.5% radiochemical purity, which was determined by paper chromatography in two solvent systems, CHCl₃:ethanol:ammonia, 80:10:1 and CHCl₃:95% ethanol:ammonia, 10:85:25.

Male Sprague-Dawley rats (180–220 g) were killed by cervical dislocation and decapitated, and their brains were removed immediately. Samples of brain tissue were homogenised in 100 volumes of 0.05 M sodium potassium phosphate buffer (pH 7.4) containing 0.01% Triton X-100 using a Polytron ultraspeed homogeniser. In the standard binding assay, aliquots (10–100 µl) of a striatal homogenate (0.01–0.1 mg protein) were added to 2 ml of phosphate buffer containing 1 µM ¹⁴C-fluphenazine (13,500 c.p.m.) alone or in the presence of 100 mM dopamine or 0.1 mM unlabelled fluphenazine. The mixture, in a 20-ml glass beaker, was incubated at 37°C for 10 min and then poured into a polypropylene centrifuge tube. The beaker was then washed with 1 ml of phosphate buffer. After centrifugation at 40,000g for 10 min, the supernatant fluid was decanted and the pellet was rinsed with 10 ml of phosphate buffer. Bound radioactivity was extracted into 1 ml of ethanol and 10 ml of dioxane–naphthalene liquid scintillation fluid and assayed using a Beckman LS 355 liquid scintillation counter. Counting efficiency for ¹⁴C was 65%.

Displaceable ¹⁴C-fluphenazine-binding was obtained by subtracting from the total bound radioactivity the amount not displaced by high concentrations of dopamine (100 mM) or fluphenazine (0.1 mM). In a typical assay measuring the binding of ¹⁴C-fluphenazine to a sample of striatal homogenate (0.05 mg protein), 2,400 c.p.m. of ¹⁴C-fluphenazine was bound, of which 70% was displaced in the presence of excess dopamine or unlabelled fluphenazine. The same maximal displacement of bound ¹⁴C-fluphenazine was obtained with excess nonradioactive fluphenazine or dopamine.

Displaceable ¹⁴C-fluphenazine-binding was linear between 0.01 and 0.1 mg of striatal protein (Fig. 1) and was saturable with increasing concentrations of ¹⁴C-fluphenazine. Half-maximal binding of ¹⁴C-fluphenazine to samples of striatal homogenate (0.05 mg protein) occurred at 4.9 µM unlabelled fluphenazine (Fig. 1), as compared with half-maximal displacement by unlabelled fluphenazine, which occurred at 4.6 µM. As half-maximal saturation occurs at nearly the same concentrations of unlabelled and ¹⁴C-fluphenazine, this indicates that ¹⁴C-fluphenazine is biologically equivalent to the nonradioactive drug in terms of tissue binding. The binding of ¹⁴C-fluphenazine in the presence of excess dopamine or nonradioactive fluphenazine was not saturable and increased linearly with increasing ¹⁴C-fluphenazine or tissue concentration.

The optimal pH for displaceable ¹⁴C-fluphenazine binding was 7.4, with a steep decline in binding at more acid and alkaline

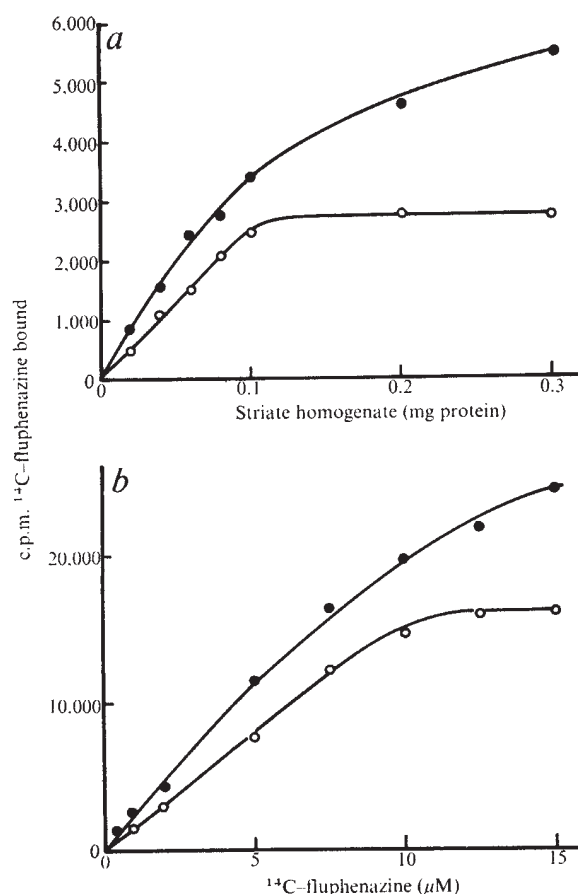


Fig. 1 *a*, Tissue linearity of displaceable ¹⁴C-fluphenazine binding to components of homogenates of the striate region of rat brain. Dilutions of a 1 in 100 homogenate of striatal tissue incubated with 1 µM ¹⁴C-fluphenazine alone or in the presence of 100 mM dopamine in the standard binding assay. Displaceable ¹⁴C-fluphenazine binding was obtained by subtracting from the total bound radioactivity the radioactivity bound in the presence of dopamine. Each value is the mean of triplicate determinations, with variations of less than 10%. *b*, Binding of ¹⁴C-fluphenazine to striatal homogenates as a function of the concentration of fluphenazine. The binding of various concentrations of ¹⁴C-fluphenazine alone or in the presence of 1 mM nonradioactive fluphenazine to samples of striatal homogenate (0.05 mg protein) were measured using standard assay procedure. Displaceable ¹⁴C-fluphenazine binding was estimated by subtracting from the total bound radioactivity the radioactivity bound in the presence of excess unlabelled fluphenazine. Each value is the mean of triplicate determinations, which differed from one another by less than 10%. ●, Total, ○, displaceable.

values. Displaceable binding occurred at incubation temperatures from 4 to 40°C, with a rapid decline at higher temperatures. If, however, striatal homogenates were subjected to 100°C for 10 min, cooled and then incubated with ¹⁴C-fluphenazine at 37°C, the displaceable fluphenazine-binding was reduced by only 30%. This suggests that the temperature dependence of the incubation step is a result of the instability of fluphenazine and catecholamines at higher temperatures rather than destruction of binding sites. Nondisplaceable ¹⁴C-fluphenazine-binding was not dependent on either pH or temperature. High concentrations (5–100 mM) of Na⁺, K⁺, Li⁺, Ca²⁺ or Mg²⁺ in the incubation medium did not affect displaceable fluphenazine-binding.

The association and dissociation of displaceable ¹⁴C-fluphenazine binding to striatal homogenates as measured by the rate of displacement by 1 mM unlabelled fluphenazine was rapid, being fully equilibrated within less than 2 min. Reliable determinations of binding at intervals less than 1 min require a

Table 1 Subcellular distribution of ^{14}C -fluphenazine binding in the striate region of rat brain

	^{14}C -fluphenazine bound (c.p.m. per mg protein)	
	Total	Displaceable
Whole homogenate	37,960	27,640
Crude nuclear pellet (P_1) (1,000g, 10 min)	45,020	29,740
Crude mitochondrial pellet (P_2) (17,000g, 20 min)	60,360	41,720
Crude microsomal pellet (P_3) (100,000g, 1 h)	55,400	37,460
Osmotically shocked P_2 subfractions		
Mitochondrial myelin pellet (9,000g, 20 min)	25,620	13,260
Crude synaptic membrane pellet (48,000g, 20 min)	64,380	41,740

Tissues were prepared and subjected to differential centrifugation. A sample of P_2 pellet was resuspended in 20 volumes of ice-cold, distilled water and homogenised with a ground glass homogeniser and centrifuged for 20 min at 9,000g. The supernatant fluid was collected and the pellet, a bilayer with a soft buffy upper coat, was rinsed with the supernatant fluid to collect the upper layer. The supernatant fluid was then centrifuged at 48,000g for 20 min. The pellets obtained were suspended in 0.5 M sodium potassium phosphate buffer (pH 7.4) containing 0.01% Triton X-100. The data shown represent the mean of four experiments in which the variations among the results were less than 15%. Protein was determined by the method of Lowry *et al.*⁹ with bovine serum albumin as the reference standard.

more rapid separation technique, such as filtration, in order to separate tissue from the incubation media more quickly; the high degree of binding of ^{14}C -fluphenazine to various filter materials prevented the use of such a technique.

Endogenous levels of dopamine are highest in the striate region of the brain, in which most of this amine is localised in dopaminergic nerve terminals⁷; therefore, if displaceable ^{14}C -fluphenazine binding is specific for dopamine receptors, regional studies of the brain should indicate more binding to homogenates of the striate region than to other portions of the brain. Moreover, subcellular studies may be expected to indicate that the greatest binding to the crude mitochondrial fraction, which contains 'pinched off' nerve endings and the associated postsynaptic membrane, in which the neurotransmitter receptor sites are thought to be localised. The crude mitochondrial fraction prepared from the striate region of the brain exhibits most displaceable ^{14}C -fluphenazine binding, but other subcellular fractions, in particular the crude microsomal pellet, also have extensive binding capacity (Table 1). After the crude mitochondrial pellet was subjected to hypo-osmo-

tic shock, the displaceable ^{14}C -fluphenazine binding was restricted primarily to the subfraction containing synaptic membranes. This suggests that the displaceable ^{14}C -fluphenazine-binding sites are consistent with postsynaptic dopamine receptors in the striate region; however, regional studies of the brain revealed a rather uniform pattern of displaceable ^{14}C -fluphenazine binding (Table 2). Although most binding occurred in the striate region and the cerebral cortex, there was no correlation between the regional concentration of dopamine and the displaceable ^{14}C -fluphenazine binding capacity. The displaceable binding to peripheral organs and tissues was much less than that observed in brain tissue, with the liver and a sample of striated muscle having the lowest binding capacity of the tissues studied. The regional and subcellular distributions of displaceable fluphenazine binding were similar when the concentration of ^{14}C -fluphenazine in the incubation medium was reduced to 0.5 μM , the limit of sensitivity of the assay system.

Table 3 Relative potencies of drugs in reducing displaceable ^{14}C -fluphenazine binding to homogenates of the striate region of rat brain

Drug	ED ₅₀ (μM)	No effect (mM)
Fluphenazine	4.6	Acetylcholine 100
Thioridazine	8.4	GABA 100
Chlorpromazine	32	Glutamic acid 100
Haloperidol	35	Glycine 100
Spiroperidol	41	Pargyline 1
Clozapine	62	Iproniazid 1
Pimozide	380	Pentobarbitone 10
Promethazine	430	Thiopentone 10
Imipramine	450	Diazepam 10
Benztropine	460	Chlordiazepoxide 10
Propranolol	500	Dopa 100
Dichlorisoprenaline	600	Homovanillic acid 100
Tranlycypromine	900	3-Methoxy-4-hydroxyphenyl-glycol 100
Dexchlorpheniramine	1,000	Adrenochrome 100
Phentolamine	1,200	Atropine 1
Phenoxybenzamine	1,900	
Methysergide	4,800	
<i>d</i> -Amphetamine	7,500	
Clonidine	17,000	
<i>l</i> -Noradrenaline	28,000	
Apomorphine	29,000	
Dopamine	30,000	
6-Hydroxydopamine	34,000	
<i>l</i> -Adrenaline	35,000	
Histamine	40,000	
Tyramine	42,000	
Serotonin	42,000	
Isoprenaline	44,000	
Normetanephrine	51,000	
<i>d</i> -Adrenaline	55,000	
Metanephrine	61,000	

The values shown represent the mean of two or three log-probit determinations using four concentrations of the drug.

Table 2 Distribution of ^{14}C -fluphenazine-binding to brain regions and selected peripheral tissues of the rat

	^{14}C -fluphenazine bound (c.p.m. per mg protein)	
	Total	Displaceable
Spinal cord	39,800 $\pm$ 2,980	21,500 $\pm$ 1,990
Medulla oblongata-pons	44,400 $\pm$ 3,240	28,860 $\pm$ 2,240
Cerebellum	43,400 $\pm$ 4,010	28,560 $\pm$ 2,640
Midbrain	40,200 $\pm$ 3,860	24,880 $\pm$ 2,280
Hypothalamus	40,000 $\pm$ 3,940	24,280 $\pm$ 2,140
Thalamus	34,700 $\pm$ 3,260	20,020 $\pm$ 1,960
Hippocampus	40,200 $\pm$ 2,760	24,400 $\pm$ 1,840
Corpus striatum	44,200 $\pm$ 2,870	30,580 $\pm$ 2,160
Cerebral cortex	48,200 $\pm$ 3,160	30,980 $\pm$ 2,450
Heart-atrium	10,680 $\pm$ 1,180	4,400 $\pm$ 320
Heart-ventricle	23,400 $\pm$ 1,960	10,960 $\pm$ 870
Aorta	22,700 $\pm$ 2,080	3,340 $\pm$ 310
Liver	13,750 $\pm$ 1,160	980 $\pm$ 110
Lung	9,300 $\pm$ 840	1,860 $\pm$ 160
Kidney	23,300 $\pm$ 2,210	9,180 $\pm$ 460
Small intestine	13,500 $\pm$ 1,310	6,800 $\pm$ 540
Striated muscle	3,710 $\pm$ 380	680 $\pm$ 80

The data shown represent the mean $\pm$ s.e.m. of triplicate determinations of two experiments.

In most regions of the brain the predominant biogenic amines are noradrenaline and serotonin and in all brain regions and subcellular fractions of the striate or cerebellum these amines could displace bound ^{14}C -fluphenazine with potencies very similar to that of dopamine (Table 3). Other putative neurotransmitters, such as acetylcholine, γ -aminobutyric acid (GABA), glutamic acid and glycine, were inactive in this regard. The displacement of ^{14}C -fluphenazine from binding sites by catecholamines, serotonin, their metabolites and related amines does not necessarily mean that these amines will have agonist activity at such sites; they may be very weak antagonists. This hypothesis is supported by the finding that various drugs, including α and β -receptor blockers and tricyclic antidepressant compounds, displace bound ^{14}C -fluphenazine (Table 3); in this respect they are not as potent as compounds with known antipsychotic activity. The phenothiazine antipsychotics fluphenazine, thioridazine and chlorpromazine were the most potent in displacing bound ^{14}C -fluphenazine, whereas pro-

methazine, a phenothiazine with weak antipsychotic properties, was about 100 times less active than fluphenazine. All compounds tested that possessed antipsychotic activity could displace bound ^{14}C -fluphenazine in the striate region and cerebellum and there was a potency correlation between these two effects. A similar correlation exists between the clinical efficacy of antipsychotic drugs and their ability to block the activation of adenylyl cyclase by dopamine in selected brain regions^{1,2}. Although dopamine did have affinity for fluphenazine binding sites, the variety of biogenic amines and related compounds that could displace bound fluphenazine in all brain regions suggested that dopamine receptors were not specifically labelled at the concentration of fluphenazine used, which is higher than that required to block the dopamine receptor of adenylyl cyclase¹. Therefore, although my studies support indirect evidence that antipsychotic drugs may act by blockade of receptor sites for biogenic amines, they also suggest that this action is not confined to striatal dopamine receptors at postsynaptic sites.

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Received July 26; revised September 9, 1974.

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Tyrosinase in the skin of albino hamsters and mice

MEASUREMENTS of tyrosinase (EC 1.10.3.1) in animals with oculocutaneous albinism have led to contradictory conclusions. Using the incorporation of ^{14}C -DL- or L-tyrosine into melanin, one investigator found no, or questionable, activity in albino mouse skin^{1,2} while another³ reported very low, but significant activity in particles from albino mouse eyes treated with Triton X-100. When the production of ^3HOH from 3,5- ^3H -L-tyrosine was used as an assay, albino hamsters⁴ were found to have no detectable activity, while albino rats⁵ were reported to have minimal, but real activity. We report clear evidence for a tyrosine hydroxylase with tyrosinase characteristics in the skin of two strains of albino hamster and one strain of albino mouse. The method used permits quantitative comparison with tyrosinase in pigmented skin. A distinct dopa oxidase is present in extracts of albino mouse.

Tyrosine hydroxylating activity of tyrosinase was measured⁴ by incubating (at 37° C) 3,5- ^3H -L-tyrosine, 1 μmol (2 to 3 $\times 10^5$ d.p.m.); L-3,4-dihydroxyphenylalanine (dopa), 0.15 μmol ; sodium phosphate (pH 6.8) or sodium pyrophosphate (pH 7.4) 20-30 μmol ; and enzyme in a total volume of 1.25 ml. Where indicated in the Tables dopa was omitted. When diethyldithiocarbamate (DDC) was added the components were incubated together for 1 h at 37° C before addition of tyrosine and dopa. The blanks were reactions without enzyme; blanks have been subtracted from observed values to obtain the values reported (averages

Table 1 Tyrosine hydroxylation by skin homogenates from TWA albino and Syrian golden hamsters

Experiment and type of hamster	Dopa (1.2×10^{-4} M)	DDC (1×10^{-3} M)	^3HOH (nmol h ⁻¹ g ⁻¹ skin)
(1) TWA albino	+	—	23
	—	—	21
	+	+	23
	—	+	26
	+	—	372
(2) Syrian golden	—	—	30
	+	+	40

In both experiments skin from 7-d-old hamsters was homogenised in 0.02 M sodium pyrophosphate (pH 7.4) (1 g skin per 3 ml). The standard assay mixture for tyrosine hydroxylation was used except, where indicated, dopa was absent and DDC was added. The blanks were equivalent to 9.0 nmol h⁻¹ g⁻¹ (experiment 1) and 60 nmol h⁻¹ g⁻¹ (experiment 2).

of duplicates). 3,5- ^3H -L-tyrosine was purified by high voltage paper electrophoresis⁶ and ^3HOH in the stored ^3H -tyrosine was removed by evaporating the solution to dryness immediately before use. Boiled homogenates gave the same results as non-enzymatic controls.

Another assay for tyrosinase was based on the oxidation of dopa to dopachrome observed spectrophotometrically at 475 nm (ref. 7). A unit of tyrosinase is the amount catalysing the formation of 1 μmol of dopachrome min⁻¹.

Two strains of albino hamsters were used (Lakeview Hamster Colony, Newfield, New Jersey). TWA is an oculocutaneous albino with no black areas while MHA is similar but the adults develop pigmented skin in the ears. AKR/J albino mice were obtained from the Jackson Laboratory. The animals were killed between ages 5 and 7 d, either by decapitation or quick freezing. The body skins were frozen until used.

Antiserum against homogeneous hamster melanoma tyrosinase⁸ was raised in rabbits. The antiserum was titred as follows. Antiserum (0.10 ml, diluted with 10% normal rabbit serum (NRS) made up in 0.05 M sodium borate (pH 8.5)) was incubated at 37° C for 1 h with tyrosinase (0.02-0.2 U) and sodium phosphate (pH 6.8), 30-60 μmol , in a volume of 1 or 2 ml. The solution was then centrifuged at 105,000g for 1 h and a portion of the supernatant was assayed for tyrosinase by either the ^3HOH or the dopachrome method. The enzyme remaining in the supernatant

Table 2 Dopa dependency and DDC inhibition of tyrosine hydroxylation by supernatant fractions of skin from TWA and MHA albino hamsters

Experiment and type of hamster	Dopa (1.2×10^{-4} M)	DDC (1×10^{-3} M)	Time of incubation (h)	^3HOH (nmol g ⁻¹)
(1) TWA albino	+	—	6	8.3
	—	—	6	0.8
	+	+	6	0
(2) TWA albino	+	—	6	13.3
	+	+	6	0
	+	—	3	9.2
(3) MHA albino	—	—	3	2.5
	+	+	3	0
	+	—	6.5	11.7
(4) MHA albino	—	—	6.5	6.3

Homogenates of albino hamster skin were prepared as described in Table 1. After centrifugation at 105,000g for 1 h the supernatants were dialysed against 0.003 M sodium phosphate (pH 6.8) and stirred with calcium phosphate gel (1.75 mg gel per mg protein) for 1 h. The mixtures were centrifuged at 10,000 r.p.m. for 20 min and the supernatants were assayed. The standard assay procedure for tyrosine hydroxylation was used, with dopa absent and DDC included where appropriate. Blanks were equivalent to 5.8 nmol g⁻¹ (experiment 1), 4.0 nmol g⁻¹ (experiment 2), 7.5 nmol g⁻¹ (experiment 3), and 4.6 nmol g⁻¹ (experiment 4).

was always compared with a control incubated only with NRS. No loss of activity was seen in control samples.

The tyrosine hydroxylating activity of TWA hamster albino skin (Table 1) was at a level which was missed previously⁴. Table 1 also shows that hydroxylation by TWA homogenates was not dependent on dopa and not inhibited by DDC as was tyrosinase in homogenates of pigmented hamster skin. Similar results were obtained from homogenates of MHA albino skin. Centrifugation of his homogenate at 105,000g for 1 h gave a supernatant which had 10–15% of the tyrosine hydroxylation activity. Treatment of these supernatant fractions with calcium phosphate gel gave fractions which were dependent on dopa and inhibited by DDC, properties typical of tyrosinases (Table 2).

The AKR/J albino mouse skin homogenate had a tyrosine hydroxylating activity of 0.2 $\mu\text{mol h}^{-1} \text{g}^{-1}$, independent of dopa and not inhibited by DDC. Although little or no activity was released by homogenisation in buffer alone, 10–25% of the activity was extracted with Igepal CO-630 (nonylphenoxypoly (ethyleneoxy) ethanol; GAF, New York.) The data in Table 3 show that the activity in the calcium phosphate fraction was inhibited by DDC and that in the ammonium sulphate fraction was dependent on dopa.

An immunological demonstration that tyrosine hydroxylation belonged to a tyrosinase-like protein was based on the use of antibody against tyrosinase. This is clearly shown in Table 4. The activity in skin from each of the three albino strains was bound to antibody and sedimented during centrifugation. This led to the loss of tyrosine hydroxylation activity from the supernatant.

Tyrosinase possesses both tyrosine hydroxylating and dopa oxidising activities, the latter property leading to the formation of melanin. Surprisingly, it was possible to separate a dopa oxidase from the tyrosine hydroxylase as follows. Supernatant from a AKR/J homogenate, 10 ml (equivalent to 3.3 g skin), prepared with Igepal, was loaded on a 2x5 cm column of DEAE-cellulose or CM-cellulose. The resin was washed with 0.003 M sodium phosphate (pH 6.8), 150 ml (fraction 1, containing most of the Igepal) and 0.1 M sodium phosphate (pH 6.8), 160 ml (fraction 2). The fractions were concentrated under N_2 about 15-fold with an Amicon PM-10 filter. Qualitatively the results from both resins were the same. Fraction 1 from each column retained the tyrosine hydroxylating activity, while fraction 2 had none. While fractions 1 formed some pigment, however, the fractions 2 became quite dark during incubation, indicating that these fractions retained most of the dopa oxidase activity. In one experiment, 0.8 ml of fraction 2

Table 3 Dopa dependency and DDC inhibition of tyrosine hydroxylation by supernatant fractions of skin from AKR/J albino mice

Experiment	Dopa (1.2×10^{-4} M)	DDC (1×10^{-3} M)	³ HOH (nmol g ⁻¹)
(1) Calcium phosphate gel fraction	+	—	180
	+	+	5.0
	—	—	162
(2) Ammonium sulphate fraction	+	—	44.4
	—	—	13.8

AKR/J albino mouse skin was homogenised as in Table 1 in buffer containing 1% by volume of Igepal. After centrifugation at 105,000g for 1 h, the supernatant was treated with calcium phosphate gel as described in Table 2. The supernatant was then treated with ammonium sulphate to 80% saturation. On centrifugation of this mixture the protein precipitate remained suspended with Igepal at the top of the tube. This protein-Igepal mixture was separated and dissolved in 0.05 M sodium phosphate (pH 6.8) to give a milky suspension which was dialysed against the same buffer. In experiment 1 the calcium phosphate gel fraction was used; in experiment 2, the ammonium sulphate fraction. The standard assay for tyrosine hydroxylation (6 h incubation) was used with dopa absent and DDC included where appropriate. Blanks were equivalent to 15 nmol g⁻¹ (experiment 1) and 3.5 nmol g⁻¹ (experiment 2).

Table 4 Binding of tyrosine hydroxylating activity in extracts of TWA and MHA albino hamster and AKR/J albino mouse skin to anti-tyrosinase serum

Experiment	Extract source	Equivalent amount of skin (g)	Anti-serum	NRS	³ HOH in aliquot counted (d.p.m.)	Activity lost from supernatant (%)
(1)	TWA	4.0	—	+	476	
			+	—	197	
	MHA	4.0	—	+	1,732	
(2)			+	—	0	100
	AKR/J	0.8	—	+	483	
			+	—	157	77
	Hamster (0.01 U) melanoma		—	+	9,480	
			+	—	4,675	51

Calcium phosphate gel extracts of skin from TWA albino hamsters (experiment 1) and MHA albino hamsters, AKR/J albino mice, and hamster melanoma⁹ (experiment 2) were incubated with antiserum to hamster melanoma tyrosinase (final concentration 1:3,500) or an equivalent amount of NRS. The solution contained 2 mg (experiment 1) or 4 mg (experiment 2) bovine serum albumin, sodium phosphate buffer (pH 6.8), 60 μmol , in a final volume of 1.14 ml (experiment 1) or 1.24 ml (experiment 2). The incubation was for 1 h at 37°C followed by 16 h at 4°C and then centrifugation at 100,000g for 1 h. Supernatant (0.5 to 0.8 ml) was assayed for residual tyrosine hydroxylating activity by the standard method. Time of incubation was 6 h for the albino skin extracts and 3 h for the melanoma tyrosinase. Blank values were 400 d.p.m. (experiment 1) and 555 d.p.m. (experiment 2).

oxidised 1.8 nmol dopa min⁻¹ but the fraction had no tyrosine hydroxylating activity. Hamster melanoma tyrosinase, possessing the same dopa oxidase activity, hydroxylated 16 nmol tyrosine h⁻¹.

An inhibitor of the dopa oxidase reaction of tyrosinase was present in fraction 1—0.40 ml completely inhibited dopa oxidation by melanoma tyrosinase.

Skin from newborns of all three albino animals can hydroxylate tyrosine at a rate 2–3% that for the corresponding pigmented variety (Syrian golden hamster⁴ and C57BL/6J mouse⁹). The amount solubilised by homogenisation alone (hamster) or by Igepal treatment (mouse) amounts to about 10% of that assayed in the homogenate. Further purification gives fractions which are dopa-dependent, inhibited by DDC and bound by anti-tyrosinase serum. Whether all the activity in the homogenates relates to tyrosinase cannot be decided yet. It seems unlikely that any of it is the result of a tetrahydropteridine-dependent tyrosine hydroxylase because even the crude adrenal medullary enzyme¹⁰ exhibits an absolute requirement for a pteridine cofactor. The absence of dopa-dependency could be explained by the presence of elevated levels of dopa or some other nonspecific reducing agent. The lack of inhibition by DDC cannot be readily explained. One investigator³ inhibited albino eye tyrosinase with 1×10^{-2} M DDC, while another⁹ reported success with albino skin using 2×10^{-4} M DDC.

Triton treatment did not further stimulate the activity probably because freeze-thawing had already disrupted melanosomal membranes¹¹. We were, however, also unable to confirm with skin, Hearing's observation³ that albino eye homogenates are stimulated by Triton to a level equal to that of pigmented eye.

A distinct dopa oxidase is present in albino mouse skin. The tyrosine hydroxylation fraction still retains some dopa oxidase activity, but the presence of a dopa oxidase inhibitor in that fraction complicates the picture. It is uncertain how these findings relate to human albinism. Hair bulbs from one type of human albino¹² develop slight pigmentation when they are incubated for long periods with tyrosine.

Inhibitors of tyrosinase have been reported in albino eye³ and many other tissues¹³. Their physiological role, however, remains obscure.

This research was supported in part by a grant from the National Cancer Institute.

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Received July 10; revised September 13, 1974.

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Monosynaptic excitation of motoneurons from secondary endings of muscle spindles

SINCE Lloyd's original work on the myotatic unit more than 30 yr ago¹, it has been assumed that the monosynaptic excitation of motoneurons from receptor nerve fibres derives entirely from the Group Ia afferents, which terminate as primary endings in the muscle spindles. Group Ib afferents from tendon organs are thought to exert their inhibitory actions² over di- or trisynaptic pathways³, while Group II and III fibres, which include those from the secondary endings of muscle spindles, form part of the flexor reflex afferent (FRA) system⁴, the synaptic actions of which are thought also to be mediated polysynaptically⁵. Whereas the monosynaptic excitation of motoneurons by Group Ia fibres has been directly confirmed in terms of unitary synaptic potentials evoked by single, stretch excited primary endings⁶, knowledge of the synaptic actions of fibres innervating secondary endings is known only indirectly from electrical stimulation of Group II fibres and is not without controversy⁷.

Here, avoiding the use of electrical stimulation, we have investigated the synaptic actions exerted on thoracic motoneurons by afferent fibres from primary and secondary endings of intercostal muscle spindles excited by stretch. We show that contrary to all previous beliefs, monosynaptic excitation of motoneurons can derive from secondary as well as primary endings.

Cats anaesthetised with sodium pentobarbitone, paralysed with gallamine triethiodide and artificially ventilated were prepared for intracellular recording from expiratory motoneurons of the thoracic spinal cord at T8 as previously described⁸. One or several intramuscular nerve filaments supplying the internal intercostal muscle of T8 were freed but left in continuity⁹. Afferent impulses recorded from them were used to trigger a signal averager thus extracting from the intracellularly recorded synaptic noise averaged synaptic potentials evoked by individual receptor nerve fibres⁸.

All signals were recorded on magnetic tape, which enabled subsequent detailed analysis of the synaptic potentials evoked by a large number of afferent fibres using the method shown

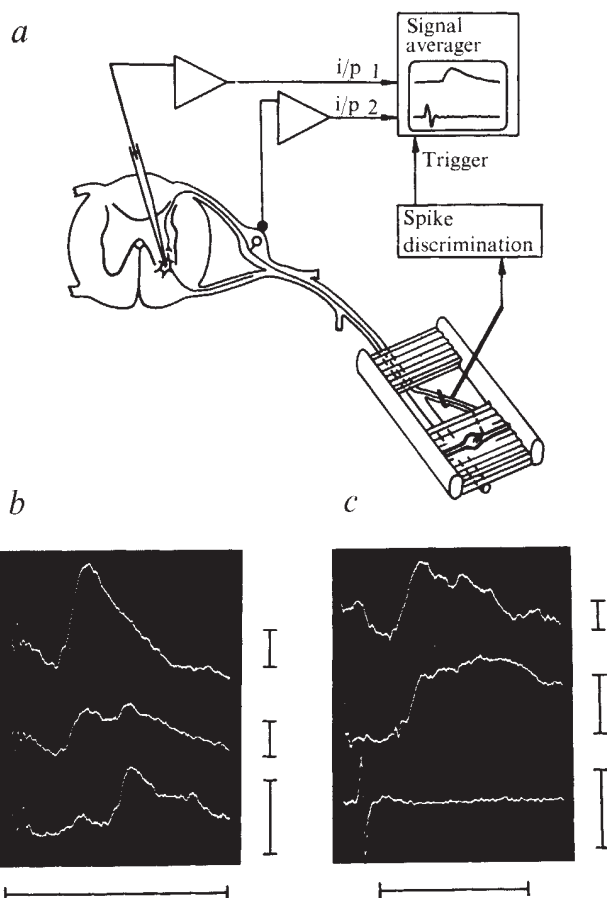


Fig. 1 *a*, Experimental set-up. *b*, Spike-triggered average responses in an expiratory motoneurone. Three different 'window' settings of the spike discriminator, using as triggers from above down, large, medium and small amplitude spikes. Upper two traces, averages of 8,192 sweeps; lowest trace, average of 16,384 sweeps. Conduction distance, 47 mm to the ventral horn. *c*, Averaged responses evoked in two different expiratory motoneurons by the same, single unit trigger spike, together with its action potential averaged from the surface of the dorsal root ganglion (lowest trace). This unit showed a low dynamic sensitivity and a very regular firing pattern. Upper trace, average of 1,024 sweeps; lower two traces, averages of 8,192 sweeps. Conduction distances: 29 mm to the ganglion and 43 mm to the ventral horn. Calculated conduction velocity of the afferent, 38 ms⁻¹. Calibrations, 5 ms and 10 μ V, except *c*, 1 μ V for lowest trace.

schematically in Fig. 1*a*. The filament recording was fed to a 'window' discriminator so that spikes of given amplitude ranges could be selected as triggers for the averager. Analysis was carried out by repeated replaying of the tape, each time with a different setting of the 'window', so as to obtain a sequence of records such as illustrated in Fig. 1*b*. Typically, the response consisted of a composite synaptic potential which could be broken down into unitary synaptic potentials each defined by a specific latency, having a fast rise-time (10–90% in 1.0 ms) and a maximum amplitude for a given setting of the 'window'. We interpret such a response as indicating that each filament contains several receptor nerve fibres monosynaptically connected to the motoneurone concerned. This interpretation depends on the assumption that a polysynaptic pathway would result in a synaptic potential with a much slower rise-time in the averaged record because of variability in the firing times of the relevant interneurons¹⁰.

The individual latencies of the unitary synaptic potentials, together with an assumed value for the synaptic delay (0.5 ms) give the conduction velocities of the individual fibres concerned, and these have ranged from 24 ms⁻¹ to 90 ms⁻¹,

thus including the range given for afferent fibres from intercostal secondary as well as primary endings by Andersson, Lennerstrand and Thoden¹¹. An error in the estimation of conduction velocity could arise, however, from slowing in the intraspinal, preterminal region of the afferent fibre¹². An independent measurement of conduction velocity was therefore obtained by recording on a separate tape channel the signal from the exposed surface of the dorsal root ganglion at T8, so that simultaneously averaged recordings were obtained of the action potentials in individual fibres at the ganglion and of the synaptic potentials they evoke (Fig. 1c). Control recordings made in a similar fashion distal to the ganglion showed that the ganglion recording gave reliable estimates of the peripheral conduction velocities. The resulting measurements gave conduction velocities which remained in agreement with both ranges of Andersson *et al.*¹¹. Of 17 fibres giving monosynaptic excitation, seven had velocities above 60 ms⁻¹ and three between 60 and 40 ms⁻¹; the remaining 6 had velocities below 40 ms⁻¹ and fell completely within the range for secondary endings given by Andersson *et al.*¹¹. Individual fibres were shown to excite monosynaptically more than one motoneurone, as illustrated for the secondary ending of Fig. 1c.

All clearly distinguishable units in the filaments were sensitive to stretch and most fell into two classes. They displayed either a high dynamic sensitivity with a relatively irregular discharge or a relatively low dynamic sensitivity with a regular firing pattern, corresponding to reported properties of primary and secondary muscle spindle endings^{13,14}. Both classes were represented among those afferents giving monosynaptic excitation. This further supports our conclusion that monosynaptic excitation of thoracic motoneurons derives from both primary and secondary muscle spindle endings.

These results have several important implications which here can be dealt with only briefly. First, motoneurons participating in the intercostal stretch reflex must draw monosynaptic excitation from secondary as well as primary endings. This stretch reflex, which has been demonstrated in cats under a variety of experimental conditions (including anaesthesia) and in conscious man, has been directly implicated in the servo control of intercostal muscles¹⁵. Second, the relatively delayed excitation by secondary endings could provide an explanation for the long latency of the human intercostal stretch reflex, although such an explanation does not in itself account for the absence of early servo correction by the Group Ia afferents¹⁶⁻¹⁸. Third, because of their monosynaptic connections, the secondaries, like the primaries, are favourably sited to undergo sprouting in the advent of degeneration of central nerve fibres also terminating directly on motoneurons. This postulated growth of stretch activated synapses could be the major factor in the increased resistance to stretch of spastic muscles.

Finally, we suggest that further application of these techniques could provide direct evidence to support the hypothesis of Matthews^{19,20} that secondary endings contribute excitation to the soleus stretch reflex of the decerebrate cat and thus to refute contrary evidence derived from electrical stimulation of Group II fibres.

This work was supported by a grant from the National Fund for Research into Crippling Diseases.

Note added in proof: We have now extended these experiments to the triceps surae muscle and find the same result, that clearly identified afferents from secondary endings monosynaptically excite homonymous motoneurons. This constitutes the direct evidence which we postulated above would support Matthews' hypothesis. Our experiments so far have been on anaesthetised preparations.

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Received August 19, 1974.

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Comparison of the number of anti-L-binding sites on LK goat red cells with the number of Na-K pumps

SHEEP¹, goats², possums³ and some other species are unusual mammals in that the red cells of individual animals have either high (HK) or low (LK) concentrations of potassium, which correlate with the activity of the Na-K pump. The pump of LK cells is less active than that of HK cells^{4,5}. When LK sheep red cells are injected into HK sheep an antibody of the IgG class (anti-L) is raised which, when reacted with LK sheep or goat red cells, stimulates the pump^{5,6}. In goat cells the major, if not the only, effect of the antibody is to reduce the affinity of the pump for K at the inside cell surface, and that stimulation results from the relative increase in the effectiveness of Na as a substrate. It has been reported that in sheep, anti-L also increases the maximal pump rate⁸. The antibody might function by binding either to the pump, and so altering its kinetic characteristics, or to another component of the cell surface, indirectly altering the environment of the pump. If it combines with the pump, the number of anti-L molecules bound should be about the same as the number of pumps. We have now confirmed this using an estimate of the number of pumps of LK goat cells obtained with ³H-ouabain⁹, and our own estimate of the number of ¹²⁵I-labelled antibody molecules bound when the pump is stimulated maximally. We used LK goat red cells as LK sheep red cells seem to contain a second antigen in addition to the antigen associated with pump stimulation^{5,10}.

The antiserum used in these experiments was obtained from one HK sheep after immunisation with cells from a single LK sheep. No haemolysing antibodies for the red cells of the HK and LK goats used in this study were present in the serum. The antiserum was absorbed against cells from three HK sheep. The methods used for determining the number of antibody-binding sites were similar to those used in determining the number of anti-D binding sites on human red cells¹¹. IgG was purified by

Table 1 Binding of anti-L by HK and LK goat red cells

Antibody quantity (mg)	Molecules bound			Fractional pump stimulation	
	HK	LK-1	LK-2	LK-1	LK-2
2.2×10^{-3}	8	41	42	1.83	1.96
0.7×10^{-3}	3	17	16	1.12	1.24

HK and LK goat red cells were exposed to purified antibody at 32°C for 0.5 h, the cells were separated from the antibody by centrifugation and washed in the cold, and then separated into two parts one of which was used for the determination of the number of ^{131}I -labelled molecules bound and the other for the determination of the ouabain sensitive K influx in quadruplicate. It was determined that maximal stimulation was established during a 0.5-h incubation of LK cells with anti-L, and that none of the antibody was eluted from the cells during a 1-h incubation at 0°C (as judged by the pump-stimulating effect of the antibody). Fractional pump stimulation was calculated as the pump rate in the antibody-treated cells divided by the pump rate in control cells. Similar results were obtained at five other antibody concentrations.

ammonium sulphate precipitation and repeated chromatography on DEAE cellulose; immunoelectrophoresis against anti-sheep serum revealed a single band. The IgG was iodinated by the iodine monochloride method¹²; protein labelled with ^{131}I was diluted with IgG labelled with nonradioactive I. Labelled anti-L was absorbed on cells from one of the LK goats in the presence of a large excess of unlabelled nonimmune sheep IgG. The cells were washed and haemolysed, and the antibody was eluted from the ghosts with butanol¹³, the purified antibody was diluted with a large excess of unlabelled nonimmune sheep IgG and dialysed against phosphate-buffered saline. LK and HK goat cells were exposed to the purified antibody for 0.5 h at 32°C , washed, and part of the cells was then used to determine the number of ^{131}I -labelled anti-L molecules bound and another part was used for the determination of the ouabain-sensitive K influx⁷. Solutions used during exposure of the cells to antibody and measurement of K influx have been described⁷.

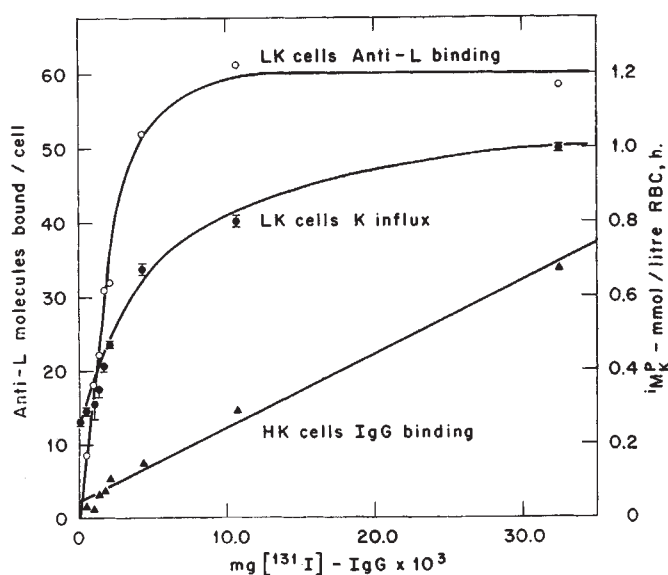


Fig. 1 The number of ^{131}I -IgG molecules bound to HK goat red cells ($\blacktriangle$) and the number of anti-L molecules bound to LK goat red cells ($\circ$) (calculated as the total number of ^{131}I -IgG molecules bound to the LK cells less the number bound to HK cells at the same antibody concentration) plotted against the number of mg of labelled IgG to which the cells were exposed (the total volume of solution containing the antibody was 5 ml for all points except the last, for which it was 7.5 ml). The simultaneously determined ouabain-sensitive K influx (iM_K) in the LK cells is also plotted ($\bullet$, right abscissa, $\pm$ s.e.m., $n = 4$) as a function of the amount of antibody to which the cells were exposed. Exposure of these cells to anti-L IgG labelled with nonradioactive I at a ratio of 1 ml of cells to 5 ml IgG (80 mg protein ml^{-1}) resulted in a pump rate of 0.885 ± 0.011 mmol per 1 red blood cells per h. The curves were drawn by eye; the HK line is a least squares plot, $r = 0.994$.

At equal concentrations of labelled antibody, cells from the two LK animals took up about the same amount of antibody (Table 1). The number of labelled molecules taken up per cell by the HK cells was a significant percentage of the number taken up by the LK cells; this uptake was thought to represent nonspecific absorption of labelled antibody¹⁴. Since the total number of antigen sites is so low on LK cells, the elution process (which presumably removes both specifically bound and non-specifically bound IgG) does not result in a monospecific antibody preparation. In calculating the number of anti-L molecules bound to LK cells, the number of molecules bound to HK cells at each antibody concentration was subtracted from the total number bound to LK cells. Since the number of anti-L molecules bound to the LK cells from each animal is about the same, it is unlikely that there are any antibodies present to antigens other than that associated with pump stimulation.

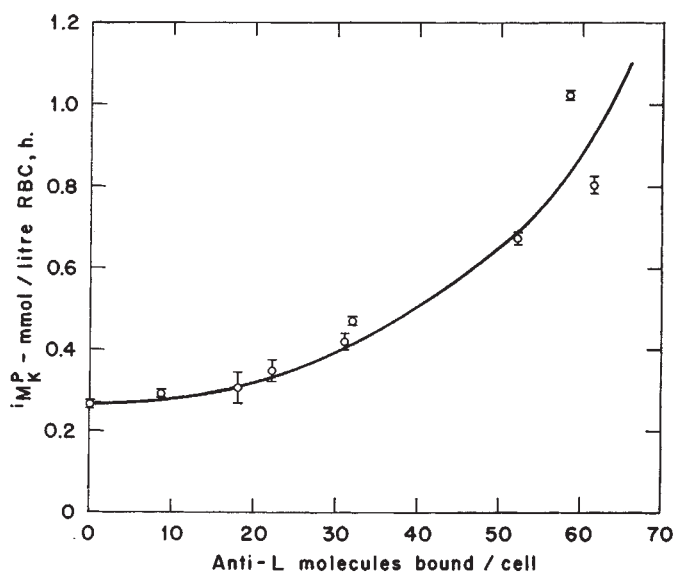


Fig. 2 The ouabain-sensitive K influx in LK cells (iM_K) is plotted as a function of the number of anti-L molecules bound per cell; the data are from Fig. 1.

In Fig. 1 the number of antibody molecules bound to HK cells and the number of corrected anti-L molecules bound to LK cells is plotted as a function of the amount of labelled antibody to which the cells were exposed. The pump rate determined on the same LK cells is also plotted as a function of the amount of antibody. The number of molecules bound to HK cells is a linear function of antibody concentration (as would be expected if this represents nonspecific binding), but the number of anti-L molecules bound to LK cells saturates at about 60 per cell which is close to the number (55) of pumps calculated from ^3H -ouabain binding to the same cells⁹. As the number of anti-L molecules bound approaches saturation, the stimulated pump rate also approaches saturation. It was not possible to determine the equilibrium constant for the antigen-antibody reaction since the eluted antibody contained large amounts of non-anti-L IgG.

In Fig. 2 the pump rate is plotted as a function of the number of antibody molecules bound. The stimulation of the pump rate is not a linear function of the number of antibody molecules bound; rather, the pump rate increases more rapidly at high antibody concentrations (high number of molecules bound) than at low. This may reflect heterogeneity in the population of LK pumps so that the pumps with higher affinities for antibody are less stimulated than are those with lower affinities or it may indicate that more than one anti-L molecule per pump must be bound before the pump is stimulated. Since similar heterogeneity has been observed in the affinity of LK pumps for ouabain⁹, the former possibility seems more likely.

Since the number of anti-L molecules bound to LK goat cells when the pump is maximally stimulated by anti-L (about 60) is

the same as the number of ^3H -ouabain molecules bound by the same cells at 100% inhibition of the pump (about 55), it is concluded that anti-L combines with the pump (or with an antigen present in the same amount as the pump). Alteration of the kinetic characteristics of LK pumps by anti-L probably results from a conformational change induced in the pump by its combination with the antibody.

This work was supported by grants from the National Institutes of Health and a grant in aid from the American Heart Association. D.L.K. was a postdoctoral fellow of the National Institutes of Health, and J.R.S. is an established investigator of the American Heart Association.

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Received June 4; revised July 15, 1974.

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Reaction of human smooth muscle antibodies with human blood lymphocytes and lymphoid cell lines

ANTIBODIES to smooth muscle appear in serum from cases of active chronic hepatitis^{1,2} and transiently in various acute viral diseases^{3,4}. The smooth muscle antibodies (SMA) are demonstrated by indirect immunofluorescence (IFL) using cryostat sections of rat stomach and kidney and human thyroid⁵. The reaction of SMA-positive sera with lymphoid tissue has been pointed out^{6,7}. Here we deal with the reaction of SMA in indirect IFL on human peripheral lymphocytes and especially with lymphoblastoid cell lines.

Lymphocytes were purified from defibrinated blood by gelatin sedimentation. Phagocytic cells were removed with iron powder and erythrocytes lysed by 0.83% NH_4Cl solution. After washing in saline the lymphocytes were cultured with or without the addition of phytohaemagglutinin (PHA) (Burroughs Wellcome), 2.5 μg per 10^6 cells ml^{-1} , in RPMI-1640 medium enriched with 10% human AB serum. After 18 h culture, the medium containing PHA was removed and new medium was added. Cell preparations were made at varying intervals as is shown in Table 1. After three washes in saline, the cells from each tube were resuspended in 0.05 ml of 0.034 M sodium citrate, smeared, dried and fixed in dry acetone at -20°C . Preparations from four different donors were examined by IFL.

Cells from lymphoid cell lines were grown in suspension culture in RPMI-1640 medium supplemented with 15% foetal

Table 1 Immunofluorescence staining of surface microvilli on smears of cells with smooth muscle antibodies (SMA)

Cell type	% Cells with short surface microvilli*	% Cells with long surface microvilli*
Moore (4 experiments)	27–64	3–20
MOLT-4 (3 experiments)	5–15	—
HeLa	—	—
L cells	—	—
Normal peripheral lymphocytes†		
before culturing	4	—
after 3 h in medium	4	—
after 18 h in medium	7	—
after 42 h in medium	20	—
after 66 h in medium	8	—
after 3 h of PHA-stimulation	5‡	—
after 18 h of PHA-stimulation	43‡	—
after 42 h of PHA-stimulation	53	1
after 66 h of PHA-stimulation	65	4

* Percentages are based on counting of 200 cells.

† One representative experiment reported.

‡ Most cells aggregated. Free-lying cells and cells in small aggregates counted.

calf serum. Two lines of Epstein-Barr virus (EBV) transformed lymphocytes, Robinson-7481 and Moore-7002, and one leukaemia cell line MOLT-4 (ref. 8), were tested. After three washes, cell smears were prepared in sodium citrate as described above. Cell preparations from nonlymphoid cell lines of human (HeLa cells) and mouse origin (L cells) were prepared in a similar manner. SMA-positive sera were allowed to react with the cell preparations using indirect IFL. The anti-human Ig-conjugate and the optic system used have been described earlier⁶.

Ten different SMA-positive sera were tested on lymphoblastoid cell lines and all gave the same type of reaction. Only the reactions of one representative serum 2888/73, will be reported here. This serum has been described earlier⁶. All SMA reactions of this serum were removed by absorption with an extract of smooth muscle from pig stomach⁶. Extracts for absorption were also made from lymphoblastoid cell lines, HeLa and L cells. One part of packed cells was mixed with two parts of phosphate-buffered saline and homogenised in an Omnimixer for 8 min. The supernatant obtained after centrifugation at 3,000 r.p.m. for 15 min was used as antigen for absorption. The

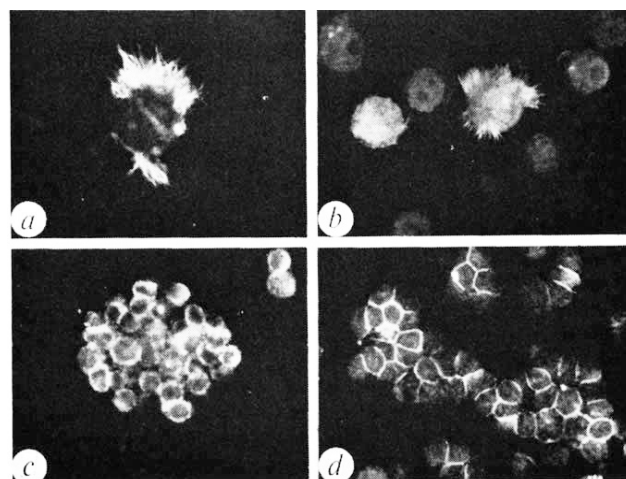


Fig. 1 *a*, Indirect immunofluorescence using SMA-positive serum on acetone fixed smear of a lymphoblastoid cell (Robinson-7481). Note the strongly fluorescent long microvilli. *b*, Indirect immunofluorescence using SMA-positive serum on acetone-fixed smear of lymphoblastoid cells (Moore-7002). Note one cell with cytoplasmic fluorescence and one cell with fluorescent microvilli and cytoplasmic fluorescence. *c*, Indirect immunofluorescence using SMA-positive serum on acetone fixed smears of peripheral blood lymphocytes cultured for 3 h with PHA. *d*, Indirect immunofluorescence using SMA-positive serum on acetone fixed smears of peripheral blood lymphocytes cultured for 3 h. ($\times 295$).

Table 2 Effect of absorption of SMA-positive serum 2888/73 with different organ or cell extracts

Absorption with extracts of:	Protein concentration (mg ml ⁻¹)	SM in rat stomach	Serum titre against:			Smears of Moore cells
			Rat kidney glomeruli	Human thyroid membrane	Mouse thymus	
Muscle layer of pig stomach:						
undiluted	19.8	<10	<10	<10	<10	<10
diluted 1/4	5.0	<10	<10	<10	<10	<10
Robinson cells	15.0	10	10	<10	<10	<10
Moore cells	15.9	<10	<10	<10	<10	<10
L cells	20.3	160	160	40	80	160
HeLa cells	17.2	160	160	80	80	80
Titre of unabsorbed serum	—	160	160	80	80	320

antiserum 2888/73 was absorbed twice with equal volumes of the different cell extracts (Table 2).

A prerequisite for the demonstration of the microvilli of lymphoid cells was that the cells were suspended in 0.034 M sodium citrate before the preparations were made or that chelating agents were added.

SMA-positive serum showed by IFL a cytoplasmic or a membrane type reaction with lymphoblastoid cells. The most conspicuous finding was, however, the strongly fluorescent long 'hairy' microvilli extending from the surface of single cells (Fig. 1a and b), usually asymmetrically concentrated to one side of the cell. Most cells with long fluorescent microvilli showed very little cytoplasmic staining. The microvilli could reach the length of the diameter of the cell but they were usually shorter or around 1/3 of the diameter. The appearance of the surface microvilli seemed to vary somewhat with the cell cycle and with the growth conditions of the cells (Table 1). MOLT-4 cells had in comparison with cells belonging to the Moore or Robinson lines only short stub-like villi and fewer cells were positive (Table 1). Myeloma cells from an IgE producing line (obtained from Dr Kenneth Nilsson, Uppsala) showed no reaction with SMA.

Wet preparations of living lymphoblastoid cells were examined by phase contrast microscopy. Almost all cells had microvilli. Attempts to stain live suspended cells with SMA-positive serum by IFL were, however, negative.

Smear and acetone fixed HeLa cells and L cells showed no microvilli stained with SMA-positive serum but a moderate or weak reaction of the peripheral part of the cytoplasm or of the membrane especially when the cells were in aggregates. This was considered to be a kind of contact phenomenon.

Absorption of serum 2888/73 with SM antigen from pig stomach removed all IFL staining of lymphoblastoid cells and also the SMA pattern on the tissue sections. The outcome of the absorption experiments is shown in Table 2. Extracts of Moore cells were almost as effective as extracts of the muscle layer of pig stomach. Extracts of HeLa cells and L cells hardly changed the titre of serum 2888.

When SMA-positive sera were set to react in IFL with smears of peripheral lymphocytes from normal blood donors a moderate reaction was observed with the cytoplasm or the membrane of 60–80% of the cells. About 5% of the cells had short villi (Table 1). When cells had been cultured for 3 h in a medium containing PHA, the cells in the aggregates formed were strongly positive (Fig. 1c). Cells cultured without PHA showed a weaker membrane-like staining (Fig. 1d) best visible when the cells were in close contact. At 18 h the IFL staining of the aggregated cells, cultured in the presence of PHA, was stronger than after 3 h. At 42 and 66 h many of the aggregates had broken up and many large and small cells with short microvilli could be seen (Table 1). Occasionally cells with long and asymmetric villi were observed resembling the lymphoblastoid cells described above. A similar increase in villi-containing cells has been observed in transmission EM after PHA stimulation¹¹.

'Hairy' cells have been observed earlier by phase contrast using wet preparations of cultured lymphoid cells, possibly pure B cell lines⁹. By scanning electron microscopy (SEM) lymphoid B

cells were shown to have long villi whereas T cells (MOLT-4) had small stub-like projections¹⁰. In the present investigation by IFL, the T cell line MOLT-4 and the probable B cell lines Moore and Robinson also showed differences in morphology, the latter cells having more and longer villi staining with SMA-positive serum than had the MOLT-4 cells.

We have demonstrated that the SM antigen present in the peripheral lymphocytes increases considerably when the cells are stimulated by PHA. Surface microvilli appear stainable by SMA-positive serum. Such surface microvilli are present in high amounts in EBV-transformed lymphoblastoid cells. We suggest that the demonstrated SM antigen, probably consisting of contractile protein(s)¹², in the microvilli are of biological importance for the lymphoid cells, for example, in their mobility. The cell lines Robinson-7481 and Moore-7002 were provided by Dr George E. Moore, Buffalo. This work was supported by the Swedish Cancer Society. K.L. received a grant from Anders Otto Swärds foundation.

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Received July 22; revised August 28, 1974.

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Replication of oncornavirus-like particle in human breast carcinoma cell line, MCF-7

THE search for RNA-containing human breast cancer viruses has been helped by the discovery of several biochemical and biophysical characteristics of the oncornavirus group^{1–4}. Although these properties may not be unique to oncornaviruses⁵, they provide biological reagents for more discriminating tests^{6–8} to establish species of origin, natural history and

virus taxonomy for candidate viruses exhibiting these properties.

Oncornavirus-like particles have been detected in human milk¹⁻⁴ and in breast carcinomas^{8,9}. Evidence for oncornavirus infection in cultures of normal breast cells¹⁰, and breast carcinoma cells¹¹ has also been reported. To date, however, undefined cultural exigencies have precluded the general usefulness of established mammary carcinoma cell lines for growing particles to levels sufficient for characterisation as human breast cancer viruses. It should be noted that replication of the mouse mammary tumour virus is, in many cases, also severely restricted in monolayer cultures of mouse mammary tumour cells^{12,13}. This restriction can be largely prevented by cultivating mouse mammary tumour cells in conditions permissive for the expression of normal mammary cell differentiative functions¹².

To obtain optimum culture conditions for the replication of a putative human breast oncornavirus, MCF-7 human breast carcinoma cells were cultivated so as to maintain several activities of differentiated mammary epithelial cells. The presence of specific 17 β oestradiol receptors¹⁵ and synthesis of α lactalbumin (W. W. Rose and C. M. McG., unpublished) suggest the expression of differentiative features of mammary epithelium by MCF-7. MCF-7 is an established subtetraploid cell line originally derived from a pleural effusion of a female patient with malignant adenocarcinoma of the breast¹⁴. Several criteria were used to establish the human nature of the cells¹⁴. These cells are free of mycoplasma¹⁴. Here we describe the isolation of an oncornavirus-like particle from MCF-7 cells and the results of immunofluorescence studies to characterise the particle serologically.

The density distribution of RNA-containing particles released from MCF-7 cells during a 48 h ³H-uridine labelling period at 25° C is shown in Fig. 1a. The buoyant density of the major particle population was determined to be 1.17 g ml⁻¹ (± 0.01) in preformed sucrose gradients. A second, minor population, with a density of 1.23–1.25 g ml⁻¹, was also detected in some experiments. These particles, which represented 1–20% of the total d.p.m. in gradients, have yet to be fully characterised. When cells were labelled at 37° C instead of 25° C, however, only particles with densities of 1.23–1.25 g ml⁻¹ were recovered.

From the 1.16–1.18 g ml⁻¹ region of 15–65% sucrose gradients in fifty different experiments 5×10^3 – 10×10^3 d.p.m. were recovered. This corresponds to a yield of 5×10^3 – 10×10^3 d.p.m. 734B/25 $\times 10^6$ cells per 48 h in our labelling and purification conditions (Fig. 1a).

RNA was extracted from ³H-uridine-labelled, isopycally-banded particles and was sized by gel electrophoresis. Four RNA populations were identified in the 734B ($\rho = 1.16$ – 1.18) particle population (Fig. 1b). The major RNA species had a molecular weight of 10×10^6 – 12×10^6 . The molecular weight of a second heterogeneous population of RNA was $\sim 3 \times 10^6$ – 5×10^6 . A third (high molecular weight) population ($\sim 1 \times 10^6$ –

2×10^6) coelectrophoresed with 28S (molecular weight 1.75×10^6) ribosomal RNA from MCF-7 cells and probably reflected microsomal contamination in the gradient. The fourth population was heterogeneous low molecular weight RNA. Parallel sedimentation velocity analyses of 734B RNA also resolved four populations, 62–70S, 35–38S, 26–28S and 4–10S, which correspond well with molecular weight estimates of 734B RNA in sizing gels.

Particles isopycally banded as above, were assayed for reverse transcriptase activity using endogenous RNA as template. The products of a 1 h 37° C reaction are shown in Fig. 2. The major RNA species consistently served as template for the polymerase reaction. Recovery of a discrete product (molecular weight 3×10^6 – 5×10^6) was variable but products

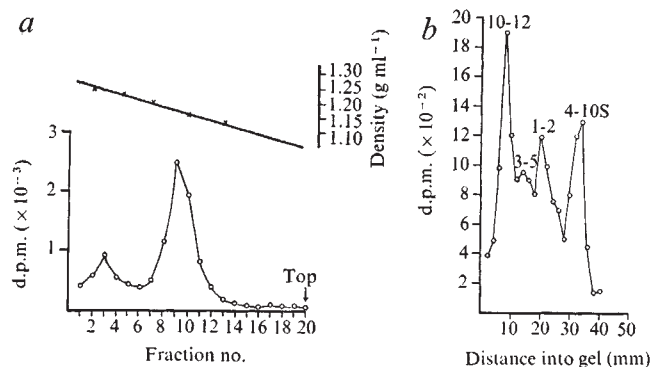


Fig. 1 a, Buoyant density of 734B particles in sucrose. Immediately before labelling 25×10^6 MCF-7 cells with ³H-uridine (15 μ Ci ml⁻¹, New England Nuclear, specific activity = 20 Ci mol⁻¹), growth medium (Eagle's MEM+10% calf serum (Flow Laboratories) + 10 μ g ml⁻¹ insulin) was replaced with MEM+5% heat inactivated (56°/30 min) calf serum+10 μ g ml⁻¹ insulin, and the incubation temperature lowered to 25°–29° C. After a 48 h label, particles were partially purified by a series of centrifugations in discontinuous and linear sucrose gradients¹³ prepared in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, (pH 7.4)). Trichloroacetic acid precipitable ³H-uridine d.p.m. in 0.2 ml fractions from linear 15–65% sucrose gradients after a 14.1×10^6 g minimum centrifugation of particles to equilibrium is shown. Radioactive materials were counted using a Beckman model 3380 instrument calibrated to deliver absolute activity (d.p.m., which corresponds to 4–5 $\times$ c.p.m. values). **b**, RNA extracted from 734B particles. Labelled particles released from 75×10^6 cells were isopycally banded as in Fig. 1. The 1.16–1.18 g ml⁻¹ region was pooled and pelleted in TNE buffer. RNA was extracted with sodium dodecyl sulphate (SDS) (0.75% final concentration) and phenol as described previously¹³. RNA was sized in 7.0 cm, 1.8% acrylamide/0.5% agarose composition gels²⁵ and mouse 28S (molecular weight 1.67×10^6) cell RNA was used as an external marker. Each fraction represents 2 mm gel slices. Numbers over radioactive peaks represent apparent molecular weights in millions of daltons derived from the external marker.

Table 1 Reactivity of MCF-7 cells with anti-MuMTV and anti-MuLV sera in immunofluorescence tests

Antiserum	Reactivity (% Cells fluorescing) [†]				MCF-7 cl-1	D-562
	MCF-6	BALB/c cl-5	BALB/cfc3H	MCF-7w		
113AV*	0	0	40–75	5–7	0	0
113AV absorbed with MuLV†	0	0	40–75	5–7	0	0
113AV absorbed with BALB/c cell‡	0	ND	40–75	5–7	0	0
113AV absorbed with MuMTV§	0	ND	0.1–1.0	0	0	0
Normal rabbit serum	0	0	0	0	0	0
Anti-MuLV	100	0	0	0	0	0

*113AV was prepared in rabbits against MuMTV isopycally banded from BALB/cfc3H milk. The serum was absorbed twice with BALB/c milk, then absorbed overnight in female BALB/c mice before use. A 1:4 dilution of serum was used in each indirect immunofluorescence test²⁶.

†MuLV grown in BALB/c spleen cells (MCF-6).

‡BALB/c cell is a lyophilised preparation of homogenised BALB/c mammary glands.

§MuMTV for absorption was isopycally banded from BALB/cfc3H milk. Three absorptions were required to reduce reactivity of the serum from BALB/cfc3H cells to 0.1–1.0%.

||Anti-MuLV was obtained from Flow Laboratories and contained both anti-gs-1 and anti-gs-3 reactivity²⁰. The serum was used at a 1:100 dilution.

¶Cells used: MCF-6, a leukaemic BALB/c spleen cell line¹⁹; BALB/c, a cloned derivative of a BALB/c mammary tumour cell line²⁷; BALB/cfc3H, Primary cultures of BALB/cfc3H mammary tumour cells¹³; MCF-7w, uncloned cultures of MCF-7 cells¹⁴; MCF-7 cl-1, a cloned subline of MCF-7; D-562, a human cell line derived from a pleural effusion of a patient with a throat adenocarcinoma¹⁸.

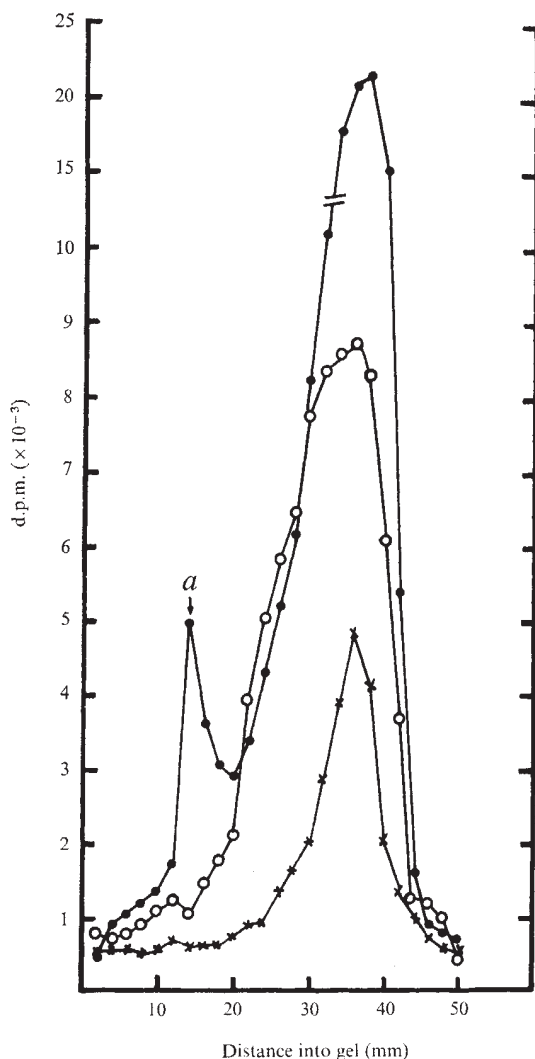


Fig. 2 Endogenous reverse transcriptase activity of 734B particles. Unlabelled particles released from 100×10^6 cells were isopycnicly banded as for Fig. 1. The $1.16\text{--}1.18\text{ g ml}^{-1}$ region was pooled and pelleted through a cushion of 15% sucrose in TNM buffer, pH 7.4 (Tris-HCl [0.01 M], NaCl [0.15 M], MgCl_2 [0.003 M]). Reverse transcriptase assays were performed¹. The incubation mixture consisted of 734B protein (130 μg), NP-40 (0.2%), DTT (0.033%), KCl (0.04 M), MgCl_2 (0.006 M), dATP, dGTP, dCTP (0.8 $\mu\text{mol ml}^{-1}$ for each) and $^3\text{H-TTP}$ (100 μCi , specific activity = 40 Ci mmol^{-1}). Reactions at 37°C were terminated after 1 h by addition of NaCl (to 0.4 M), SDS (to 1%) and phenol (to 50%). Phenol extracted products were sized in 1.8% acrylamide/0.5% agarose gels²⁵ and the molecular weight of products was determined from the position of an external 1.67×10^6 molecular weight marker (ribosomal 28S RNA). The major product (molecular weight $10 \times 10^6\text{--}12 \times 10^6$) corresponds to the radioactivity in the peak under a. ●, Products from a 1 h standard reaction¹; ○, products from a 1 h reaction incubated with pancreatic RNase (10 $\mu\text{g ml}^{-1}$) for 15 min at 37°C before addition of nucleoside triphosphates; x, products from a 1 h reaction in which deoxyguanosine triphosphate had been omitted from the reaction mixture. 734B protein (43 μg) was included in all three reactions.

ranging in size from 4×10^6 to small (4S) fragments were routinely recovered in a single heterogeneous peak which, when integrated, accounted for the vast majority of $^3\text{H-TTP}$ c.p.m. after 1 h enzyme reaction.

Preincubation of the NP-40-disrupted 734B with pancreatic ribonuclease (10 $\mu\text{g ml}^{-1}$ at 37°C) virtually eliminated the $^3\text{H-TTP}$ counts in the $10 \times 10^6\text{--}12 \times 10^6$ molecular weight region of sizing gels (Fig. 2) indicating that the high molecular weight RNA served either as template or primer in the reaction. The

lower molecular weight products were reduced by approximately 65%. Omission of deoxyguanosine triphosphate from the reaction mix also eliminated the $^3\text{H-TTP}$ c.p.m. from the $10 \times 10^6\text{--}12 \times 10^6$ molecular weight region of the gels (Fig. 2), and reduced the smaller molecular weight products by approximately 85%. This indicates synthesis of a proper heteropolymer of high molecular weight RNA-DNA rather than terminal addition of $^3\text{H-TTP}$ to RNA by a terminal transferase¹⁶.

Analysis of the reaction products in CsSO_4 gradients indicated that 15–30% of the total product after 1 h incubation consisted of RNA-DNA hybrids with a density range of $1.68\text{--}1.55\text{ g ml}^{-1}$ (Fig. 3). The remainder consisted of DNA (density ~ 1.45 , Fig. 3). That the RNA-DNA product was a hydrogen bonded heteroduplex rather than a covalently linked polynucleotide was shown by denaturing the product in formamide¹⁷ before analysis in CsSO_4 gradients (Fig. 3). Such treatment resulted in removal of the $^3\text{H-TTP}$ counts from the RNA and hybrid regions of the gradient and recovery of the counts in the DNA region of the gradient (Fig. 3).

An antiserum against murine mammary tumour virus (MuMTV) (prepared against isopycnicly banded virus from BALB/cf3H mouse milk) reacted in indirect immunofluorescence tests with 5–7% of MCF-7 cells (Table 1). Granular fluorescence was primarily localised in the cytoplasm of stained cells. A cloned derivative of the human cell line which has exhibited no evidence of 734B particle release (MCF-7 Cl-1 with no reverse transcriptase activity in a $1.16\text{--}1.18\text{ g ml}^{-1}$ gradient band) showed no reactivity with the anti-MuMTV serum (Table 1). Similarly, another human epithelial cell line (D-562), also derived from a pleural effusion¹⁸, and not associated with particle synthesis, showed no reactivity with the anti-MuMTV serum (Table 1). The antiserum did not react with BALB/c mouse cells infected with murine leukaemia viruses (MuLV) nor with MuMTV-free BALB/c mouse mammary epithelial cells²⁷ (Table 1).

Absorption of this antiserum with either MuMTV-free BALB/c mouse milk, MuLV grown in BALB/c cells or extracts of BALB/c mammary glands had no effect on its reactivity with either MuMTV positive mouse cells in primary culture¹² (BALB/cf3H) nor with human MCF-7 cells (Table 1). In contrast, absorption with MuMTV isopycnicly banded from

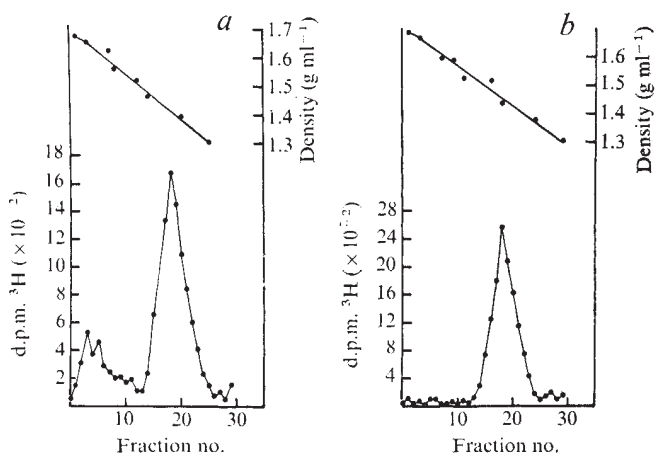


Fig. 3 Density of reverse transcriptase products in CsSO_4 gradients. Ethanol precipitated, SDS-phenol extracted product of a 1 h reverse transcriptase reaction (compare legend for Fig. 2) was further purified by the CTAB method²⁸. The product, reconstituted in $0.5 \times \text{SSC}$ buffer and 50% formamide¹⁷ (final concentrations), was divided into two equal aliquots. One half was heated at 80°C for 10 min, conditions adequate to melt RNA-DNA hybrid polymers¹⁷. Both heated and unheated products were dialysed against 0.001 M Tris buffer pH 7.0, mixed with CsSO_4 ($\rho = 1.51$) and centrifuged 60 h at 45 K in a 50 Ti rotor at 25°C . a, Unheated product; b, product heated at 80°C for 10 min.

mouse milk resulted in a marked reduction of its reactivity with both BALB/cC3H mouse cells and with MCF-7 cells (Table 1).

An antiserum against MuLV protein, reactive against both inter- and intraspecies MuLV group determinants²⁰, which in immunofluorescence tests was reactive to a dilution of 1:2,000 for MuLV positive mouse cells, was unreactive with MCF-7 cells even at a dilution of 1:100 (Table 1).

Thus, 734B particles, with a buoyant density of 1.16–1.18 g ml⁻¹, a high molecular weight RNA and a *bona fide* reverse transcriptase enzyme seem to be antigenically unrelated to MuLV. This is consistent with results of biological tests in which concentrates of 734B particles and disrupted MCF-7 cells failed to induce leukaemia (or any other overt disease) in BALB/c mice. Similarly, 734B particles did not activate defective sarcoma virus in S+L– NIH Swiss 3T3 (ref. 22) cells.

The interpretation of the antigenic relationship between 734B and MuMTV is less clear. Studies of Black and his colleagues²³ have suggested immunological cross reactivity between MuMTV and an agent in human breast carcinoma cells. Likewise, Axel *et al.*⁸ have suggested homology between MuMTV RNA and RNA from oncornavirus-like particles in human breast tumours. In our own studies, reactivity of 734B-synthesising MCF-7 cells with an anti-MuMTV serum (Table 1) coupled with the fact that the reactivity for MCF-7 cells could be absorbed from anti-MuMTV sera with purified 734B (C. M. McG., P. M. G., H. D. S., T. G., and M. A. R., unpublished), suggests an antigenic relatedness between MuMTV and 734B. Preliminary results of radioimmunoassays and protein sizing studies, however, suggest significant structural differences between MuMTV and 734B. Studies currently in progress in our laboratory to identify the protein(s) of 734B reacting with anti-MuMTV sera and to evaluate the homology between the RNAs from the two agents are aimed at the elucidation of their true relationship.

Definitive morphological characterisation of 734B requires more particles than are synthesised in existing cultural conditions. We are now attempting to stimulate 734B particle production to reproducibly quantifiable levels by altering those conditions.

We are also actively engaged in experiments to establish species of origin for 734B as well as the natural history of 734B expression in normal breast and breast carcinoma cells using the DNA product of the reverse transcriptase reaction as a 'probe'^{6,21} for 734B information. This data is of obvious fundamental importance for an understanding of the relationship of 734B to human breast cancer.

We thank Abdon Long and Anne de Sostoa for technical assistance. We also thank Dr J. Justin McCormick and Ms Lenora Larson for performing the reverse transcriptase assays and for helpful discussions. This work was supported in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Detroit, Michigan and a grant from the National Cancer Institute.

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Received June 11; revised September 16, 1974.

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Unique increase of serum proteins and action of antitumour polysaccharides

We have reported that lentinan and several other polysaccharides inhibited the growth of sarcoma-180 transplanted subcutaneously in mice^{1–4}, and that their antitumour activity is a host-mediated reaction with participation of the thymus or thymus-dependent cells (T cells)^{5–8}. While it has recently been reported that lentinan is a T cell oriented adjuvant⁹ and a helper T cell stimulant^{10–11}, it is not clear how lentinan affects the host at the first stage before the induction of many immunological changes. It is possible that the antitumour activity of lentinan is dependent on the presence of certain substances in the host which interact with these polysaccharides.

We have found that three kinds of protein components, which are different from properdin, increase markedly in mouse serum soon after lentinan administration. It should also be noted that there is a close relationship between the increase of protein components and the antitumour activity of polysaccharides.

ICR female mice (body weight 20–25 g) were given a daily intraperitoneal injection of 4 mg per kg lentinan (the optimal dose for tumour regression) for 5 d. Serum was obtained conventionally from blood on days 1, 4, 7, 10, 14, and 21 after the final injection of lentinan. A 5 µl aliquot of serum obtained on each of these days was applied to polyacrylamide gel electrophoresis on gradient gel PAA 4/30 (Pharmacia Fine Chemicals, Uppsala) at 125 V for 15 h using 5 mM Tris-glycine buffer (pH 8.6). The gel was stained with 1% solution of Amido black 10B, followed by destaining in 7% solution of acetic acid.

The results of electrophoresis show that mouse serum proteins were divided into 21 fractions (Fig. 1). It is apparent that three protein components (LA, LB, and LC) markedly increase in

Table 1 Unique increase of serum protein, LA, LB, and LC, after the administration of antitumour polysaccharides

Polysaccharides	Dose (mg kg ⁻¹ × 5)	Increase of serum proteins				Tumour inhibition ratio (%)
		LA	LB	LC	Date of peak*	
Lentinan	4	+++	+++	+++	4	98.0
Pachymaran	10	++	+++	+++	7	95.3
CM-pachymaran†	75	++	++	++	7	98.4
Zymosan	20	++	+++	+++	7	91.5
Control (saline)	0.1 (ml)	—	—	—	—	—
Pachyman	10	+	++	+	1	— 7.0
Laminarin	10	—	—	—	—	— 7.0
Starch	10	—	—	—	—	Not effective
Dextran‡	10	—	—	—	—	—21.2
Cellulose	10	—	—	—	—	10.1

The mice were ICR female mice.

* Date of peak was counted after the last injection of polysaccharides.

† Carboxymethylpachymaran.

‡ Molecular weight 200,000–275,000.

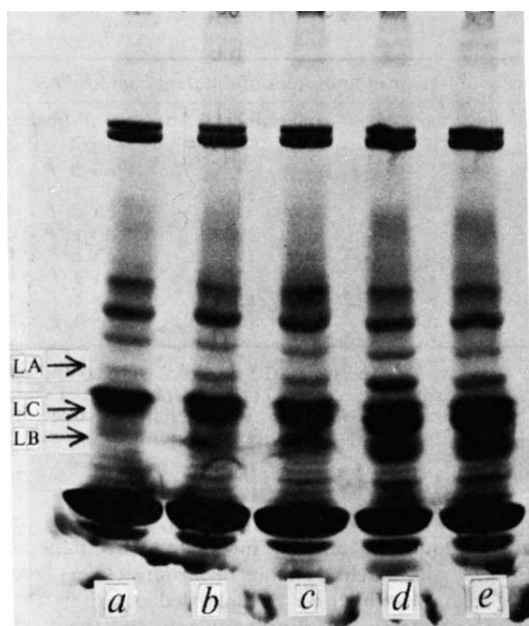


Fig. 1 The acrylamide gel pattern of serum samples from lentinan-treated mice. *a*, Control (fourth day after saline injection); *b*, first day; *c*, third day; *d*, fourth day; *e*, seventh day after the last injection of lentinan.

the serum of mice administered lentinan. Their increase peaked on days 4 to 7 after the final injection of lentinan, then gradually decreased, and the contents of LA, LB, and LC on day 21 were not different from those in control mice. Quantitative measurement of serum proteins with a densitometer showed that serum proteins LA, LB and LC on the fourth day after the last lentinan injection were 2.8, 2.4, and 2.1 times as much as those in the control serum. The ratio of LA, LB, and LC content to the whole serum contents was 6.2%, 10.3%, and 11.3%, respectively. Although the complete assignment of serum components fractionated by this method is not yet established, we consider that LA is β globin and LB and LC are α globins.

Further, we studied the correlation between the increase of serum protein components and the antitumour activity of polysaccharides. The alteration in the content of serum proteins LA, LB and LC in mice administered polysaccharides with or without the activity was compared, the method being identical to that used with lentinan. These results are summarised in Table 1. There was an increase of serum protein LA, LB, and LC in mice administered antitumour polysaccharides, such as lentinan or pachymaran, even though the duration of peak varied. On the other hand, changes of serum protein LA, LB, and LC in mice given polysaccharides without antitumour

activity was not significantly different from those in control mice, except for pachyman.

Pillemer *et al.*, reported that properdin, a β globulin, which was adsorbed from serum by incubation with zymosan, increased in titre 200–300% above the normal level 2–14 d after the intravenous injection of zymosan^{12–14}. Serum protein LA, LB, and LC, however, were not adsorbed with zymosan or lentinan by Pillemer's method. In addition, there is much more LA, LB, and LC than properdin in serum. These findings seem to indicate that none of proteins LA, LB, and LC is properdin.

That the antitumour polysaccharides have an anti-complementary activity *in vitro*¹⁵, and that they affect α -helix content of serum albumin¹⁶, indicates that in an early stage, serum factors play an important role in tumour regression, as well as in cell-mediated immune responses. We consider that there may be a close relationship between the drastic increase of the proteins (LA, LB, and LC) and a potent stimulation of T helper cells¹¹. Although the relation between these serum proteins (LA, LB, and LC) and the host defence mechanism against tumours remains obscure, the dramatic increase of these proteins by antitumour polysaccharides may indicate a new approach to the study of antitumour immunity.

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Liposomes as immunological adjuvants

ADJUVANTS are widely used to increase antibody formation in experimental animals, for example, Freund's incomplete adjuvant, a water-in-oil emulsion containing the antigen, and Freund's complete adjuvant, which is the same but with killed tubercle bacilli. These adjuvants cannot be used in man because the mineral oil base is not degraded and persists at the injection site. Particularly with the complete adjuvant, unacceptable granulomas can be formed. There is real need for a safe and effective adjuvant for use in human immunisation programmes.

Such an adjuvant could reduce the amounts of antigens,

which is administered to humans, can be conveniently labelled with radioactive iodine and is readily incorporated into liposomes; the formation of antibodies can be measured easily by passive haemagglutination. Representative observations in mice immunised with free and liposome-entrapped DT by the intravenous and subcutaneous routes are presented in Table 1. DT administered intravenously in negatively-charged liposomes elicits the formation of much higher concentrations of antibodies than are elicited by free antigen. Subcutaneous (footpad) inoculation of DT in negatively-charged liposomes elicits significantly higher primary and secondary antibody responses than inoculations of the same doses of free antigen. In contrast, antigen entrapped in positively-charged liposomes elicits less antibody than the same dose of free antigen.

Intramuscular injection of DT in negatively-charged liposomes again elicits significantly higher antibody levels than free DT (Table 1). In these and other experiments, dicetylphosphate-containing liposomes as adjuvants were superior to those containing phosphatidic acid. When immune mice were inoculated intravenously with free DT they developed severe

Table 1 Serum antibody responses of mice to diphtheria toxoid administered free or in liposomes of different compositions

Experimental group	No. of mice	Mode of administration	Route of administration	Primary Ab response	Secondary Ab response	Probability
a	15	Free	Intravenous	1.8	—	a v b, $P < 0.01$
b	15	—Liposomes (PA)	Intravenous	10.0	—	
c	6	Free	Subcutaneous	2.5	11.6	c v d, $P < 0.01$
d	6	—Liposomes (PA)	Subcutaneous	6.7	13.3	d v e, $P < 0.01$
e	6	+Liposomes	Subcutaneous	0	11.3	c v e, $P < 0.10$
f	6	Free	Intramuscular	1.7	7.5	f v g, $P < 0.10$
g	6	—Liposomes (PA)	Intramuscular	3.7	11.0	g v h, $P < 0.05$
h	6	—Liposomes (DP)	Intramuscular	6.6	12.0	f v h, $P < 0.01$

Mice were of the TO strain, except in groups c–e, which were of the CBA strain. Positive (+) liposomes (0.5 mg lipid) were composed of egg lecithin, cholesterol and stearylamine in molar ratios 7:2:1; in negative (–) liposomes phosphatidic acid (PA) or dicetylphosphate (DP) replaced stearylamine. Primary serum antibody responses were measured after 13 or 14 d, booster injections with 20 µg antigen in the same form were given and secondary responses were measured after a further 10 d (groups c–e) or 14 d (groups f–h). Antibody responses were measured by indirect haemagglutination (ref. 4) and expressed as the log₂ IH titre.

for example, diphtheria and tetanus toxoids, required for immunisation, with corresponding economies especially relevant to the developing countries. Live virus vaccines have hazards such as the potential initiation of persistent infections or malignancy, and vaccines containing killed viruses or viral antigens often require adjuvants to elicit the formation of protective antibodies. Another possible danger of the use of adjuvants is that in a minority of recipients they may induce allergic reactions to the vaccines which they contain, especially if antigens are administered twice to boost immunity, or if the recipients are already sensitised to an antigen at the time of its administration. It would be an advantage if the mode of presentation of antigens in the adjuvant were such as to avoid allergic reactions. If adjuvants consist of immunogenic materials such as glycolipids or proteins, even in trace amounts, they might themselves induce allergic or even autoallergic reactions through sharing of antigens with those of the recipient or some other mechanism.

We report here the use of liposomes, concentric spheres consisting of phospholipid bilayers separated by aqueous compartments, as immunological adjuvants. Protein and other antigens can be entrapped in liposomes, which have been proposed as carriers of therapeutic agents^{1,2}, and we have found that under certain conditions the administration of antigens so entrapped elicits the formation of more antibodies than are elicited by the same doses of free antigen. Liposomes can be made of non-immunogenic, biodegradable materials and their composition and properties such as electric charge can be varied at will. If animals which already have antibodies are challenged with antigens entrapped in liposomes they are much less likely to develop antibody-mediated allergic reactions than when challenged with the same doses of free antigens³.

Diphtheria toxoid (DT) was studied because it is an antigen

serum sickness reactions, and the majority died. In contrast, the same amount of antigen inoculated subcutaneously or intravenously did not elicit Arthus or serum sickness reactions³. The investigations which we have carried out show that liposomes are potentially useful as adjuvants and there is reason to believe that further modifications of the system, such as the incorporation of *Bordetella pertussis*, *Mycobacterium tuberculosis* or microbiological or chemical products known to have adjuvant activity, will further increase their usefulness in practice.

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Received August 19; revised September 23, 1974.

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Differences in the effects of adult thymectomy on T-cell mediated responses *in vitro*

EVIDENCE exists indicating that functionally different subsets of T lymphocytes are activated during the *in vitro* allograft response. Thus, the T cells responding primarily by proliferation in the mixed lymphocyte culture (MLC) may be distinct

from the T cells which differentiate to killer cells and display specific target cell lysis (CML)¹⁻³. The biological properties of the T-cell subsets involved are incompletely known. Adult thymectomy (ATX) in mice and rats alters certain T lymphocyte mediated functions. Spleen T cells forming rosettes with sheep erythrocytes disappear as soon as 5 d after ATX^{4,5}. Lymphocyte with high amounts of surface θ antigen are depleted from the spleen within a few weeks after the operation (Ref. 6 and C. Fournier and J. F. B., unpublished).

These early changes in the T-cell functions following ATX have been taken to indicate the existence of a newly formed, shortlived T lymphocyte population in the spleen regulated by the intact thymus either through a continuous cell traffic from the thymus or through the action of a thymic hormone⁷. We have investigated whether the proliferating and lytic functions in the MLC-CML may be distinguished by selective depletion of a thymus-dependent (T) cell population by ATX, and also tested the effect of ATX on the *in vitro* responses of mouse spleen cells to the T mitogens, phytohaemagglutinin (PHA) and concanavalin A (con A). The results indicate that the killer cell precursors are more dependent on intact thymus function than the precursors of proliferating cells.

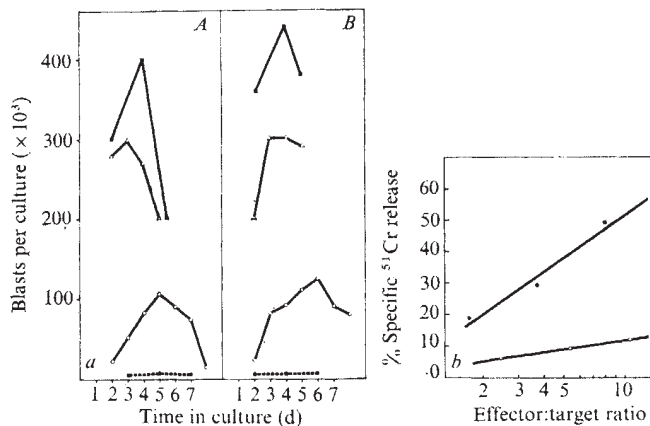


Fig. 1 *a*, Responses of CBA/H spleen lymphocytes to DBA/2 mitomycin-blocked spleen cells (O), PHA (Δ) and con A (■) 56 d after adult thymectomy. —●—●—, background responses of non-stimulated CBA/H cells. *A*, lymphocytes of ATX mice; *B*, lymphocytes of STX controls. *b*, Lysis of P-815 mastocytoma cells by CBA/H + DBA/2m-primed cells on the day 7 of culture in the MLC. O, MLC responder cells from ATX mice; ●, responder cells from STX controls (10,000 target cells, exposure time 6 h).

CBA/H-T6T6 or CBA/H mice were thymectomised by suction at 5 and 6 weeks of age. Age- and sex-matched controls were either sham-thymectomised (STX) or left untreated. Three separate experiments, each including 30–80 ATX mice were performed. Three to ninety days after the thymectomy the spleens were removed, spleen cells of three to five mice in each group were pooled and the lymphocyte-enriched population of the spleen cells (yielding approximately 85% of lymphocytes) was processed as described⁸. The spleen lymphocytes were then passed through nylon-wool columns⁹ to remove most of the non-T cells. After the procedure, 8–15% of the cells were found to carry surface immunoglobulin detectable by immunofluorescence with FITC-conjugated sheep anti-mouse Ig; 1.5×10^6 spleen lymphocytes from each group were cultured with optimal concentrations of PHA-M (Difco Pharmaceuticals, Detroit, final dilution 1:150) or con A (final dilution $10 \mu\text{g}^{-1}$ ml) in 2 ml of Eagle's minimal essential medium (Orion Pharmaceuticals, Helsinki), substituted with 5% of heat-inactivated foetal calf serum from selected batches (Flow Laboratories, Irvine, Scotland) in 11.5×100 mm round bottom test tubes (Münnerstadt Glaswarenfabrik, Münnerstadt) in a humidified atmosphere of 5% CO₂ in air⁸. Alternatively, the spleen lymphocytes were stimulated by 3.0×10^6 mitomycin-C blocked DBA/2

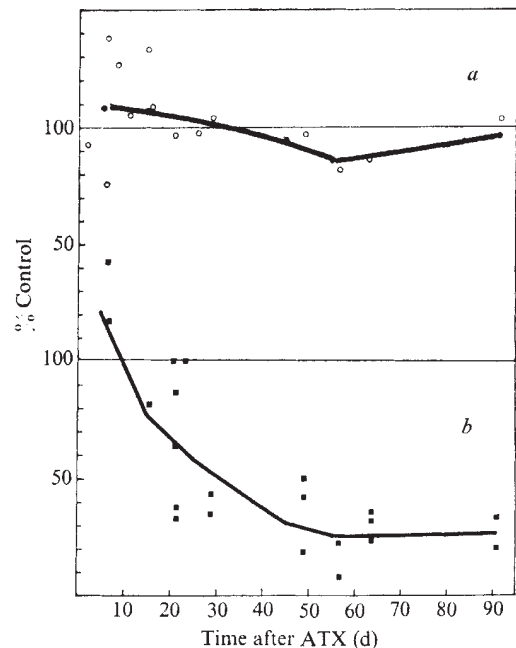


Fig. 2 Responses of CBA spleen lymphocytes to allogeneic lymphocytes (DBA/2m) in MLC (*a*) and lysis of P-815 (DBA/2) mastocytoma cells by MLC-primed cells (*b*), 3–90 d after adult thymectomy. All responses given as % responses of sham-operated control spleen cells tested on the same day. For detail see text.

cells (referred to in the text as DBA/2m) in conditions where only T cells are responsive^{10,11}. On day 7 or 8 of culture, the MLC-primed cells were tested for the ability to lyse relevant ⁵¹Cr-labelled P-815 mastocytoma target cells. In the cell-mediated lysis (CML) test⁸ standardised conditions and 6 h exposure times were employed. Specific ⁵¹Cr release was calculated according to Brunner¹², the background release being in all experiments less than 15% of maximal release.

Examples of typical mitogen, MLC and CML responses by lymphocytes from thymectomised and sham-thymectomised mice in a single test (56 d after the thymectomy) are given in Fig. 1*a* and *b*. Fifty six days after ATX, the mitogen and MLC responses were close to sham-operated controls, whereas the CML response was clearly depressed. As responses of spleen lymphocytes obtained from non-treated control mice did not significantly deviate from sham-operated controls, the non-treated controls have not been included in the results. Figure 2 summarises the pooled data of the three separate experiments. As regards the mitogen and MLC responses, only maximal responses, regardless whether they take place on the same day or not, have been considered. The responses of lymphocytes obtained from thymectomised mice are expressed as percentage responses of lymphocytes obtained from sham-operated controls. Spleen lymphocytes from ATX mice responded less efficiently to PHA from week 3 onwards after the thymectomy. The responses remained depressed for approximately 8 week, but then returned back to control level. A similar depression, taking place in approximately equal time period after ATX, was observed in the responses of spleen lymphocytes to con A. The depression, however, was not as remarkable as the depression of the response to PHA. A slight depression in the response of spleen lymphocytes to allogeneic cells was observed 7–9 weeks after adult thymectomy. This was in marked contrast with the effect of ATX on the CML response by MLC-primed cells. The ability of *in vitro* primed cells to lyse relevant allogeneic target cells was possibly first increased (up to 2 weeks ATX) but markedly depressed from the week 3 onwards, and remained depressed to approximately 30% of the control level throughout the observation period of 90 d.

The depression of T-cell responsiveness to phytohaemagglutinin, con A and in the MLC and CML reactions may be explained by a decrease in the relative amount of T cells in the spleen, especially since the percentage of spleen θ -positive cells decreases after ATX (refs 4 and 6 and C. Fournier and J. F. B., unpublished). The proportional decrease in the number of T cells, however, cannot explain our results since first, the responder cells have been passed through nylon columns before being used, which is known to remove macrophages and most non-T cells⁹ and, second, there is an obvious dissociation between the various T-cell functions quantitated. The enrichment of T cells afforded by nylon-wool filtration could explain the relatively low depression of the proliferative response in the MLC found in this study as compared to earlier reports^{13,14} (which in addition concerned two-way MLC).

Our results, as regards the temporary depression of T-cell mitogen responses following ATX, confirm the findings by Folch and Waksman on the effect of ATX on T-cell mitogen responses in the rat¹⁵. Our findings show a dissociation in the effects of ATX on the MLC and CML responses. The CML response seems to be more dependent on an intact thymus function than the MLC response. Howe has shown that anti- θ serum plus complement inhibited CML responses more readily than MLC responses, suggesting that the killer cell precursors had a larger amount of θ antigen on their surface than the MLC proliferating cell precursors¹⁶. Since ATX selectively depletes those spleen cells which express a high amount of θ antigen on their surface (C. Fournier and J. F. B., unpublished), it may be that these cells are the precursors of CML effector cells.

The FITC-conjugated sheep anti-mouse Ig was a gift from Professor A. Fagraeus, State Serum Institute, Stockholm and the con A a gift from Professor H. Wigzell, University of Uppsala.

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Ganglioside catabolism in hexosaminidase A-deficient adults

TAY-SACHS disease is an inborn error of glycosphingolipid metabolism characterised by the accumulation of N-acetyl-galactosaminyl - (N-acetylneuraminy) - galactosylglucosylceramide (G_{M2}) and N-acetylgalactosaminylgalactosylglucosylceramide (G_{A2}) in various tissues of affected individuals¹. A defect in the removal of N-acetylgalactosamine from G_{M2} , catalysed by a hexosaminidase, has been demonstrated as the underlying deficiency in all forms of G_{M2} -gangliosidosis^{2,3}. In Tay-Sachs disease this defect has been associated with the absence of one (the A form) of the two major isozymes of β -N-acetylhexosaminidase (Hex A and Hex B)⁴. Hex A can be differentiated from Hex B in serum, leukocytes, cultured skin and amniotic fluid cells, and urine by using various physical characteristics of these enzymes⁵. The smaller proportion of A relative to B is the basis of screening for heterozygous carriers of Tay-Sachs disease⁶.

A deficiency of Hex A has been demonstrated in the healthy members of a family where other members have had Tay-Sachs disease^{7,8} and this is difficult to reconcile with the currently accepted interpretation of the absence of Hex A in Tay-Sachs disease. It has been suggested⁷ that this relationship can be clarified by studying the hydrolysis of labelled G_{M2} and G_{A2} in appropriate tissues from these clinically normal A-deficient adults. We report here the catalytic properties of leukocyte hexosaminidases from such individuals when the natural substrates, G_{M2} and G_{A2} , are used to measure activity. These levels are compared with those present in normal A-containing adults and Tay-Sachs heterozygotes and homozygotes.

Leukocytes were prepared from 20 ml of blood as follows⁹. Red cells were allowed to settle from whole blood at room temperature in a heparin-dextran solution. Plasma containing leukocytes and platelets was removed and centrifuged at 1,000g for 10 min. The pellet, containing white cells, was removed and subjected to osmotic shock to remove residual red cells and then recentrifuged. The resulting pellet was washed twice with physiological saline. The final white cell pellet was homogenised in 2.5 ml of 0.25 M sucrose in a tight-fitting all glass homogeniser and the homogenate was centrifuged in the cold at 1,500g. The supernatant was removed and used as the source of enzyme. Incubations were carried out with the synthetic substrate 4-methylumbelliferyl- β -N-acetylglucosamine⁷ or the specifically labelled natural substrates G_{M2} and G_{A2} ³ with the modifications noted in Table 1.

Leukocyte preparations from normal controls, Tay-Sachs heterozygotes and A-deficient adults all possessed enzymes able to degrade the artificial and natural substrates. Leukocytes from the Tay-Sachs homozygote possessed no catalytic activity toward G_{M2} as substrate but could degrade G_{A2} . Although the specific activity of G_{M2} hydrolysis in the Tay-Sachs heterozygotes was lower than the A-deficient adults, and probably indicates some differences in the enzymes' active site, this difference in specific activity by itself may not be significant. There were no differences in the specific activities of the groups when artificial substrate was used to monitor total hexosaminidase activity. As previously shown, using the artificial substrate⁷ the A-deficient adults and Tay-Sachs homozygotes were phenotypically the same in their deficiency of the A form. All samples studied had catalytic activity toward G_{A2} with overlap in the specific activities between groups. By consideration of the ratio of G_{M2} activity to G_{A2} activity, it was possible to classify the heterozygotes as having a ratio of about one half of the normal controls. This type of classification is valid as no deficiency in G_{A2} hydrolysis is reported in Tay-Sachs homozygotes. When the same ratio was calculated for the A-deficient adults, similarities to the Tay-Sachs heterozygotes rather than to normal controls were obtained. It is believed, however, that these two heterozygous states may be fundamentally different as the lower G_{M2}/G_{A2} ratio in classic Tay-Sachs carriers seems to be due to a decrease in G_{M2} hydrolysing

Table 1 Artificial and natural substrate hydrolysis in leukocytes from normal and various A-deficient persons

Source	Hexosaminidase activities			Ratio G_{M_2}/G_{A_2} (pmol nmol ⁻¹)
	4-MU-N-acetylglucosaminide* (μ mol mg ⁻¹ protein ⁻¹)	G_{M_2} † (pmol mg ⁻¹ protein ⁻¹)	G_{A_2} ‡ (nmol mg ⁻¹ protein ⁻¹)	
Normals 1	4.3	200	3.8	53
2	4.6	230	6.4	36
3	5.2	310	8.9	35
Tay-Sachs homozygote	4.8	0	10.7	—
Tay-Sachs heterozygotes				
1	2.4	90	5.2	17
2	4.4	120	6.3	19
3	5.1	150	8.2	18
A-deficient adults				
1	4.0	180	9.8	18
2	4.8	190	16.7	11
3	4.8	220	15.2	14
Parents of A-deficient adults				
Mother	3.2	170	11.3	15
Father	4.6	280	9.7	29

*See ref. 7.

†Incubations contained 10 nmol of GalNAc-³H-G_{M₂} (16,000 c.p.m.), 50 mM citrate-phosphate buffer (pH 4.4) and extract in a total volume of 200 μ l. Incubations were for 3 h at 37° C and assay for GalNAc-³H released was as previously published³.‡Incubations contained 10 nmol of GalNAc-³H-G_{A₂} (20,000 c.p.m.) 50 mM citrate phosphate buffer (pH 4.0), 0.24 mg sodium taurocholate, and extract in a total volume of 200 μ l. Incubations were for 1 h at 37° C and GalNAc-³H released was assayed as before³.

activity, while in the A-deficient adults there is a combination of lower G_{M_2} and higher G_{A_2} hydrolysing activity. It was of interest to study the parents of the A-deficient adults who appear as Tay-Sachs heterozygotes by conventional criteria, to determine whether they might be classified by the evaluation of G_{M_2} to G_{A_2} activity. The mother with a ratio of 15 seems to be a true Tay-Sachs heterozygote; the father possesses an intermediate ratio, 29, and seems to be the carrier of a new mutation which was previously postulated by pedigree analysis.

It has been generally assumed that only Hex A is involved in G_{M_2} degradation¹⁰. Significant amounts of G_{M_2} -hydrolytic activity, however, have been demonstrated in homogeneous preparations of either Hex A or B from human placenta¹¹. In these preparations, the kinetic properties of both hexosaminidases with this substrate were essentially identical. Although it is difficult to extrapolate from these data to the situation *in vivo*, the possibility that Hex B normally participates in G_{M_2} catabolism must be considered. If this is indeed the situation, then the loss of the A form and existence of individuals with no A should be expected. One of the correlates of this interpretation is the necessity of an abnormality in Tay-Sachs patients of at least the part of the hexosaminidase B component involved in G_{M_2} degradation. The data presented here are consistent with both the genetic model previously postulated⁷ and with the model for hexosaminidase relationship already proposed^{11,12} where A is a conformer of B or represents an independent modification of the B gene product.

These findings are also consistent with the common and unique subunit theory for the relation of Hex A and B¹³ but are not readily reconcilable to the specifier subunit theory¹³.

Finally, as long as enzymatic activity with artificial substrates alone is used to classify carriers and patients with Tay-Sachs disease⁵, it will be impossible to distinguish both the A-deficient adults from those with Tay-Sachs disease and their parents from typical Tay-Sachs carriers. The frequency of this subpopulation among Tay-Sachs carriers is not known and based on current methods of prenatal diagnosis¹⁴, the A-deficient adults would today be classified as Tay-Sachs patients.

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Received August 12; revised September 13, 1974.

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Specific binding of growth hormone to thymocytes

As well as its general trophic effect on lymphoid tissue¹, growth hormone (STH) specifically increases the activity of thymocytes in a graft *versus* host reaction² and their helper activity³. *In vitro* studies showed that STH alters the mitotic activity, RNA and protein metabolism of thymocytes^{4,5}. At least for the effects obtained *in vitro*, it is assumed that STH binds to the thymocytes and thereby changes their physiological activity. The nature of this binding has not been studied. To learn about the characteristics of the surmised interaction, bovine STH (Nutritional Biochemicals, Cleveland, Ohio) and bovine prolactin (National Institutes of Health, lot NIH B4) were labelled with ¹²⁵I and their binding to lymphoid cells was studied.

The labelling mixture contained 3 mg hormone, 200 μ g lactoperoxidase (Sigma Lot No. 112c-0320), 100 μ g glucose-oxidase (Worthington, GOP 2 LA, 105 IU mg⁻¹), 5 mg glucose, 5×10^{-7} M KI and 5 mCi carrier-free ¹²⁵I (Eidgenoesische Reaktorforschungs AG, Wuerenlingen) in 2 ml 0.5 M phosphate buffer, pH 7.8. The reaction was carried

out at room temperature for 20 h with constant stirring. A slight precipitate was removed by centrifugation; STH was then precipitated by the addition of 3 ml 1 M phosphate buffer, pH 7.0, prolactin by the addition of 2.2 ml saturated ammonium sulphate, pH 7.0, the precipitates collected and redissolved in 2 ml 0.05 M phosphate buffer, pH 7.8. This procedure was repeated four more times. The final supernatant contained negligible amounts of radioactivity. The precipitate was assumed to contain only hormone, since in preliminary trials the enzymes used did not precipitate in the conditions described. The protein concentration of the final solution was determined by optical density readings at 2800 Å, and the radioactivity of an aliquot determined. From this, a specific activity of 5×10^{16} d.p.m. mol^{-1} STH was calculated. Prolactin was labelled to the same extent.

Cell suspensions were prepared from calf thymus and brachial lymph nodes, obtained from the local slaughterhouse within 15 min of killing the animal. Mouse thymus, mesenteric lymph nodes and thymus from mice injected 2 d previously with 2 mg of hydrocortisone were obtained from C3H/eb mice. The lymphoid organs were cut with scissors into fine pieces in cold Hank's balanced salt solution (BSS) and then squashed with a Teflon pestle against the walls of a loose fitting tube. Tissue debris was left to settle and the

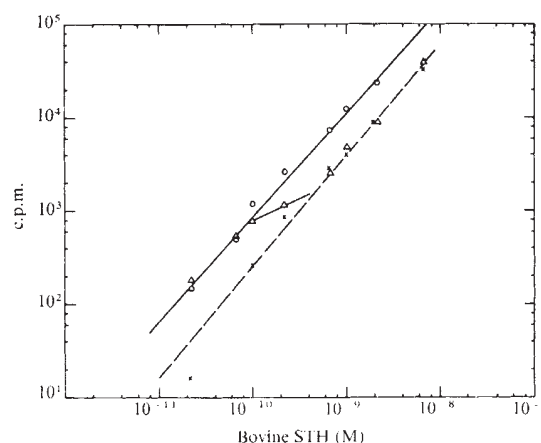


Fig. 2 Inhibition of binding of STH to murine thymocytes (TC) by pretreatment with an excess of STH or prolactin. Murine thymocytes were incubated in unlabelled hormone at a concentration of 10^7 cells per $10 \mu\text{g}$ hormone ml^{-1} (15 min at 4°C) and washed twice with BSS. The rest of the procedure was as described in the legend to Fig. 1. Δ , c.p.m. on mouse TC, preincubated in bovine prolactin; $\circ$, total c.p.m. added; $\times$, c.p.m. on mouse TC, preincubated in bovine STH.

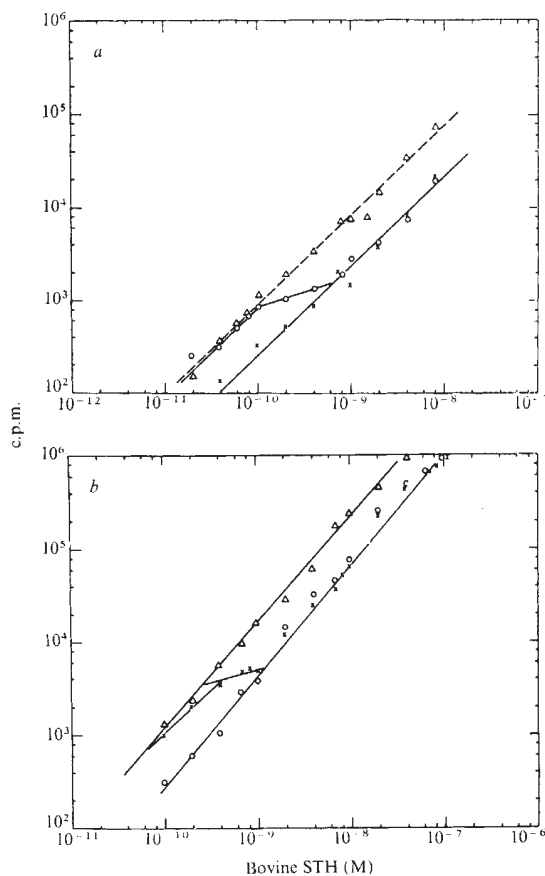


Fig. 1 *a*, Binding of bovine STH to bovine thymocytes (TC) and lymph node lymphocytes (LNC). Triplicate samples of 2.5×10^6 cells were incubated in the cold for 15 min in 2 ml Hank's balanced salt solution containing labelled hormone at the indicated concentration. They were then spun down, 1 ml of the supernatant counted in a γ counter with an efficiency of 46%. Also, 1 ml of the hormone dilutions was counted to give the total c.p.m. added (Δ). The arithmetic mean of the differences between corresponding tubes containing cells and supernatant and only supernatant is given as c.p.m. bound to cells; $\circ$, c.p.m. on bovine TC; $\times$, c.p.m. on bovine LNC. *b*, Binding of bovine STH to murine TC and LNC. Δ , total c.p.m. added; $\circ$, c.p.m. bound to mouse LNC; $\times$, c.p.m. bound to mouse TC.

supernatant cell suspension was then washed once with cold BSS. Cell viability, as estimated by trypan blue exclusion, was over 90%. Cells were then incubated with decreasing amounts of labelled hormone in the cold and spun down. The radioactivity of half the supernatant was determined and separately that of the cell pellet with the other half of the supernatant. Differences in counts obtained were taken as evidence that hormone had bound to the cells and were used to estimate the amount of hormone bound by the cells (Fig. 1). It was found that bovine and murine lymph node cells (LNC) bound, at all the concentrations tested, about one third of the total amount of either STH (Fig. 1) or prolactin added (data not shown). Hydrocortisone-resistant murine thymocytes were similar to LNC in their binding behaviour. Normal thymocytes bound about the same fraction of the hormone at concentrations above 10^{-9} M; below that, however, they took up increasing proportions of the total STH present, but not of prolactin. At about 10^{-10} M all the STH was associated with the cells. In contrast, prolactin was bound with decreasing efficiency by thymocytes at all concentrations below 5×10^{-10} M.

The binding of all the STH added to thymocytes at low concentrations could be reduced by about two-thirds by preincubating the cells in unlabelled STH (Fig. 2). Preincubation in unlabelled prolactin (Fig. 2) or thyroxine ($2 \mu\text{g}$ per 10^7 cells in 2 ml BSS, 15 min at 4°C) had no effect upon the binding of STH to either LNC or thymocytes.

It therefore seems that there are two mechanisms by which STH binds to thymocytes: one demonstrable at high concentrations of the hormone, that is above 10^{-9} M, the other at physiological concentrations. The first one would account for the binding of about 1/3 of all the hormone added; it seems to be unspecific and not saturable, since binding could not be inhibited by preincubation of the cells in unlabelled STH or prolactin. This type of binding was also observed with lymph node lymphocytes and hydrocortisone resistant thymocytes. The other type of binding, observable at physiological concentrations of STH and below, seems to be saturable and specific, since it could be inhibited by preincubation of thymocytes in unlabelled STH but not of the structurally similar prolactin⁶. This binding is probably also of high affinity, since thymocytes incubated in labelled growth hormone (10^7 cells per $10 \mu\text{g}$ STH per ml, BSS, 15 min at 4°C) will, upon prolonged washing (12 centrifugations over a period of 24 h) retain an amount of

radioactivity per cell equivalent to that found at 10^{-9} M STH in the dilution assay. In these conditions, lymph node cells shed all the radioactivity bound initially.

These data fit well with the assumption of specific receptors for STH being present in the thymocytes membrane.

The maximal amount of hormone taken up by the thymocytes through these postulated receptors can be estimated from Fig. 1, assuming saturation of the receptors at that concentration of hormone, when radioactivity first appears in the supernatant. From the specific activity of the hormone, the total number of cells in the incubation mixture, and the efficiency of counting, it may be calculated that about 10,000 molecules of STH were bound to one calf thymocyte and 20,000 to a murine thymocyte (average of three independent experiments, yielding, respectively, 18,000, 20,000 and 22,000). Whether this represents indeed the number of high affinity, specific binding sites on thymocytes is not clear as some of them may be occupied by endogenous hormone bound previously *in vivo*. The number of receptors may thus be substantially higher.

Specific binding of STH to cells has previously been demonstrated with human lymphocytes grown *in vitro*⁷. Whether the binding of STH to lymph node lymphocytes influences their physiological state is unknown. It is also unknown which part of the binding of STH to thymocytes induces changes in their metabolism *in vitro* and whether this is related to their biological activity³.

I thank Dr R. Citronbaum for help with the radioiodination procedure, Dr N. K. Jerne for his hospitality at the Basel Institute for Immunology and Dr R. Bates of the National Pituitary Agency for supplying prolactin. This work was supported by the Schweizerische Nationalfond zur Foerderung der wissenschaftlichen Forschung and the Emil Barrell-Stiftung.

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Received July 9, 1974.

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Initiation of polyphenylalanine synthesis and the action of cytokinins in fenugreek cotyledons

The initiation of peptide chain formation is thought to be the point of control in protein synthesis. Plant growth hormones may also act at this point.

Isolated cotyledons of fenugreek (*Trigonella foenum-graecum* L.) respond to cytokinins¹ and it has become evident that at an early stage the growth response in darkness is dependent on protein synthesis. It has been shown that cytokinin treatment of cotyledons increases the activity of the ribosome fraction in

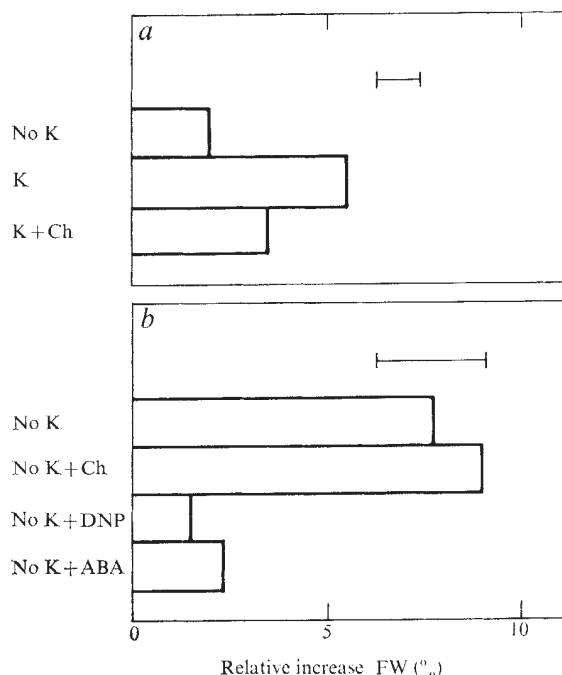


Fig. 1 Cotyledons (five per dish) were weighed before and after incubation at 25° C in darkness in Petri dishes (six replicates per treatment) on filter paper wetted with 20 mM KNO₃ and other compounds as indicated. Antibiotics used were penicillin, streptomycin and mycostatin, 25 µg ml⁻¹ each. LSD values at $P = 0.01$ are shown. *a*, Stimulation of fresh weight increase of cotyledons after 6 h application of 5×10^{-5} M kinetin (K) and inhibition of this stimulation by 10 µg ml⁻¹ cycloheximide (Ch). *b*, Inhibition of fresh weight increase after 47 h in the control (no kinetin) situation by 5×10^{-5} M dinitrophenol (DNP) and 5×10^{-7} M abscisic acid (ABA) but not by 25 µg ml⁻¹ cycloheximide.

in vitro protein synthesis². The ribosomes from treated cotyledons are more active in initiation of poly(U)-directed polyphenylalanine synthesis.

Cotyledonary petioles with enclosed plumules and radicles were removed from dry fenugreek seed by making two cuts. The cotyledon containing parts were sterilised, washed, and incubated overnight in Petri dishes on moist filter paper. The cotyledons were then isolated and subjected to a preliminary 24 h incubation in control conditions (20 mM KNO₃, 50 µg ml⁻¹ chloramphenicol) before such treatments were applied. Other antibiotics were used, however, in some experiments (Fig. 1). Neither these antibiotics nor chloramphenicol affected the growth response. From sterilisation onwards, the material was in darkness except for manipulations carried out under green safe light.

The growth response to cytokinins at its earliest observable stage seems to depend on protein synthesis. A stimulation of relative fresh weight increase (expansion) after 6 h of kinetin application is shown in Fig. 1*a*; this stimulation was reduced significantly by cycloheximide at 10 µg ml⁻¹. In contrast, even after 47 h, the 'slow expansion' in the absence of cytokinins was not inhibited by cycloheximide at 25 µg ml⁻¹, (Fig. 1*b*) or at 10 µg ml⁻¹ (ref. 3). This is noteworthy because in the control condition, protein synthesis takes place and is inhibited by cycloheximide, and because the 'slow expansion' in the control condition is severely inhibited (Fig. 1*b*) by either dinitrophenol (5×10^{-5} M) or abscisic acid (5×10^{-7} M). Therefore, cytokinin-induced growth in these cotyledons seems linked in a special manner to protein synthesis.

Significant effects on incorporation of amino acids have been observed 3, 2 and even 1 h after cytokinin application. In one experiment an increase of 48% of ¹⁴C-leucine incorporation into protein due to kinetin treatment was observed at the end of a 3 h incubation. In that experiment, cycloheximide (10 µg ml⁻¹) inhibited incorporation in both the control and kinetin treatment to 16% of the uninhibited control value. Kinetin did

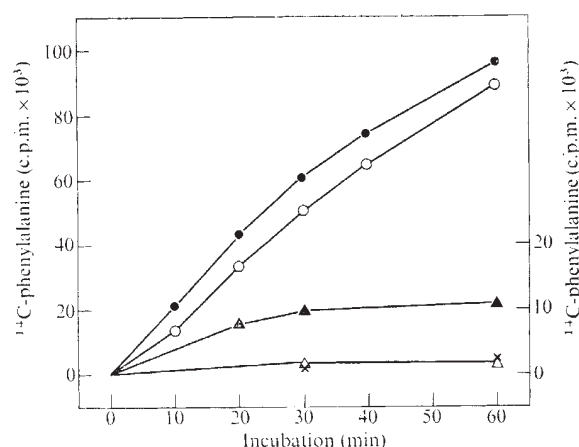


Fig. 2 ^{14}C -phenylalanine incorporation with time in an *in vitro* incorporation system without poly(U) ($\times$), with poly(U) ($\circ$), and the same but after 5 min preincubation at 30°C of tRNA, poly(U) and ribosomes ($\bullet$). Aurintricarboxylic acid ($5\ \mu\text{g}$) added at time zero (Δ) or after 5 min of preincubation on ice of tRNA, poly(U) and ribosomes ($\blacktriangle$). Extracts were prepared by grinding lots of 100 cotyledons with mortar and pestle to a final volume of 12 ml of 0.1M Bis-Tris (bis (2-hydroxyethyl) amino tris (hydroxy methyl) methane) (Sigma) HCl, pH 7.8, 40 mM KCl, 10 mM MgCl_2 , 1 mM dithiothreitol. They were then centrifuged for 20 min at $18,000g$ and after addition of 200 μl 25% Triton X-100 the post-mitochondrial supernatants for 1 h at $160,000g$. 5 ml of supernatant were dialysed for 2–3 h against 1 l of buffer; the $160,000g$ pellet was resuspended in buffer and centrifuged for 90 min at $160,000g$. The pellet was again carefully resuspended, this time in about 2.5 ml of buffer and centrifuged once more at $5,000g$ for 5 min as was the dialysed supernatant. The absorbances at 260 nm were read and corrections made for differences by dilution. Assay: 30 min, 30°C , 0.5 ml total volume, buffer 0.1 M Bis-Tris pH 7.8, KCl 40 mM, MgCl_2 10 mM, dithiothreitol 1 mM, poly(U) 50 μg , tRNA (*E. coli*) 100 μg , ATP 1.2 μmol , GTP 0.1 μmol , PEP 1 μmol , pyruvate kinase in 4 M $(\text{NH}_4)_2\text{SO}_4$ (Sigma) 1 μl , ribosomes 2.5 A_{280} units, supernatant 2.5 A_{280} units. ^{14}C -phenylalanine 0.5 μCi , (522 mCi mmol^{-1}). Stopped with TCA, heated and washed on to glass filter disks, digested with NCS solubiliser, and counted in toluene 2,5-diphenyloxazole scintillant mixture. * No poly(U).

not affect uptake but cycloheximide did; presumably this was a side effect of the inhibition of protein synthesis, as the inhibition of incorporation into protein was far in excess of that of uptake. In a repeat experiment, the 70% ethanol extractable amino acid content per cotyledon (4.7 s.e. 0.1 μmol) was not affected by the kinetin treatment.

In an earlier analysis using an *in vitro* protein synthesis system, it seemed that kinetin treatment of the cotyledons increased the activity of the ribosome fraction rather than that of the post ribosomal supernatant and its tRNA².

My *in vitro* amino acid incorporating system used washed, Triton-treated ribosome and dialysed high-speed supernatant fractions. The supernatant used throughout was derived from control cotyledons but the ribosomes were from either control or cytokinin-treated cotyledons and corrected for differences in concentration (see legend to Fig. 2).

Incorporation in the poly(U)-directed assay system proceeds linearly with time for about 30 min, or it may show an initial lag; but, if poly(U), ribosomes and tRNA are preincubated in buffer together, the initial lag in incorporation is removed when the other components of the assay system are added (Fig. 2). This suggests the requirement for an initiation step in polyphenylalanine synthesis, in which a ribosome-poly(U) complex is formed⁴. The initiation process may be stopped nearly completely and specifically in plant systems⁵ at any time by addition of $2 \times 10^{-5}\text{M}$ aurintricarboxylic acid (ATA); applied at zero time it removes the effects of poly(U) addition (Fig. 2). Although a preincubation, carried out at 0°C , did not seem to remove the initial lag, such preincubation does seem to afford

Table 1 Effect of treatment of cotyledons with various cytokinins for 3 h (experiment 1) with kinetin and inhibitors (experiment 2) on initiation activity

Treatment of cotyledons	^{14}C -Phenylalanine Incorporation (c.p.m.) after 30 min preincubation			
	With	Without	Difference	Relative value
Experiment 1 (3 h)				
Control	4,555	884	3,671	100
Kinetin $5 \times 10^{-5}\text{M}$	9,291	973	8,318	227
Zeatin $1 \times 10^{-6}\text{M}$	12,029	992	11,037	301
6-Benzyladenine $5 \times 10^{-6}\text{M}$	7,543	933	6,610	180
6-Methyladenine $5 \times 10^{-5}\text{M}$	3,760	671	3,089	84
Experiment 2				
Control	5,645	1,550	4,095	100
Kinetin $5 \times 10^{-5}\text{M}$, 3 h	10,203	1,707	8,496	207
2, 4 Dinitrophenol ($1 \times 10^{-4}\text{M}$), 20 h	4,373	862	3,511	86
2,4 Dinitrophenol ($1 \times 10^{-4}\text{M}$), 20 h + kinetin ($5 \times 10^{-5}\text{M}$), 3 h	3,916	923	2,993	73

Activity expressed as difference in incorporation of ^{14}C -phenylalanine after 30 min incubation of a poly (U)-directed system with $2 \times 10^{-5}\text{M}$ aurintricarboxylic acid added at time zero but with and without a preincubation of poly(U), tRNA and ribosomes for 5 min on ice as in Figs 2 and 3. Control supernatant was used in all incubations.

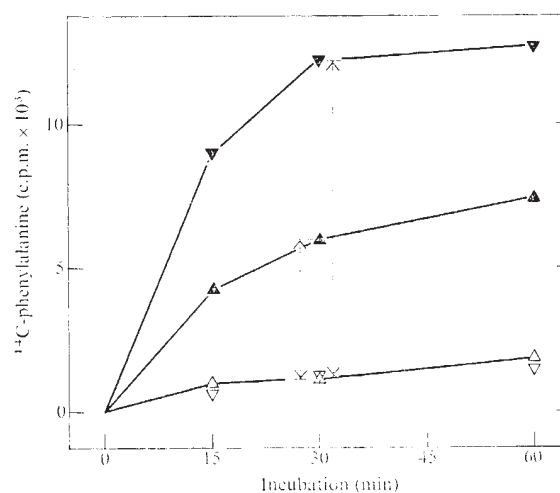


Fig. 3 Measurement of initiation activity of ribosomes from control ($\blacktriangle/\triangle$) and $5 \times 10^{-5}\text{M}$, 3 h, kinetin-treated ($\blacktriangledown/\triangledown$) cotyledons as difference (arrows) in incorporation of ^{14}C -phenylalanine in a poly(U)-directed system with aurintricarboxylic acid added at time zero but with ($\blacktriangle/\blacktriangledown$) and without ($\triangle/\triangledown$) 5 min preincubation on ice of tRNA, poly(U) and ribosomes, as in Fig. 2, but during incubation only control supernatant used.

measurable initiation activity: ATA, applied after a 5 min initiation preincubation of poly(U), ribosomes and tRNA on ice, allows some extra incorporation during the subsequent elongation-incubation period (Fig. 2).

A measure of the initiation activity is obtained by the difference between incorporations in incubations which had or had not followed preincubation of ribosomes, poly(U) and tRNA for 5 min on ice and to which ATA had been added at time zero (Fig. 3). Table 1 shows the relative values in initiation activity using ribosomes from cotyledons treated in different ways. The activity is increased considerably after treatment for 3 h with kinetin, zeatin or 6-benzyladenine, but notably not by 6-methyladenine, a base which at the applied concentration does not elicit a growth response¹.

No increase in initiation activity, however, has been observed following *in vitro* treatment of control ribosomes with zeatin (10^{-6}M) or with 6-benzyladenine ($5 \times 10^{-6}\text{M}$). The increase in activity of ribosomes from kinetin treated cotyledons is pre-

vented by the presence of 2,4-dinitrophenol for 20 h during the treatment of the cotyledons receiving kinetin during the last 3 h only (Table 1). This suggests that the kinetin effect on initiation activity requires energy.

For mammalian ribosomes, binding of poly(U) to the small subunit at 0° C has been shown to be dependent on the presence of associated protein⁶. For this protein, which is removed by a 0.5 M KCl wash, initiation-factor function has been suggested⁶. In several experiments, not detailed here, the presence of 0.5 M KCl in the buffer used for resuspending the ribosomes after the first high speed centrifugation, about halved the initiation activity of the ribosomes, which after the second high speed centrifugation were resuspended in standard buffer. A way in which the present kinetin-induced increases in initiation activity may be explained is as an increase in the concentration or as an activation of protein initiation factors.

My findings may help to explain the function of the reported binding of cytokinins to ribosomes⁷. Such binding by itself, however, does not seem sufficient to explain the observed increase in initiation activity. An improvement of the ribosome fraction has been observed for auxin treated soybean hypocotyl segments and it was suggested that this implied an enhanced level of peptide chain initiation⁸.

I thank Mrs L. Eckhardt for technical assistance and Dr J. A. Zwar for correcting the text.

Addendum: In the experiment of Fig. 2 the ribosomes were prepared from freshly dissected cotyledons as such ribosomes seem to show the lag period more readily.

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Received July 5; revised August 13, 1974.

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A receptor mediating sexual differentiation?

SEXUAL differentiation of the brain is believed to be mediated by exposure to testicular androgens at a critical period of development—prenatal in most species, but during the first 5 d after birth in rats. Only those androgens which are potential substrates for aromatising enzymes (that is, those which can be converted to oestrogens *in vivo*) are effective^{1–4}. These observations led to the concept that masculinisation of the brain is caused by local conversion of androgens to oestrogens.

There is now a considerable amount of evidence in support of this hypothesis. For example, the masculinising effects of androgens on the brain can be prevented by the previous administration of the antioestrogen MER 25 (ref. 5), and the effects of oestrogens, administered during the critical period, are similar to those of androgens^{6–9}. Aromatising enzymes are present in the hypothalamus and amygdala¹⁰ and *in vivo* conversion of ³H-androgen to ³H-oestrogen has been shown

in these areas during the critical period¹¹.

Are oestrogen receptors present in the brain during the critical period? Previous attempts¹² to answer this question have been hampered by the presence of large amounts of a specific oestrogen-binding protein (blood EBP)¹³ in the plasma of perinatal animals. Neonatal rat brain contains a similar protein (brain EBP)¹⁴ which is present in high concentrations and is possibly extracellular. It is distinguished from the oestrogen receptor of the adult brain¹⁵ by its relatively low affinity ($K_d \sim 10^{-8}$ M) for oestradiol and by the inability of diethylstilboestrol (DES) to compete with oestradiol-17 β in the binding reaction.

Here we report the use of a new and sensitive method¹⁵ to estimate the high affinity binding of oestradiol by cytosol from the brains of neonatal rats. We chose our experimental conditions so that we measured only slowly dissociating, high affinity, DES suppressible binding; the low affinity EBPs of blood and brain were not detectable.

Wistar rats (5 d old) of both sexes were used and binding was estimated in cytosols prepared from two areas of the brain: (HA) which contained both hypothalamus and amygdala, and (C) which contained cerebral cortex. Scatchard¹⁷ representations of binding isotherms (Fig. 1) were apparently rectilinear: this is consistent with the measurement of a single class of non-interacting binding sites. Dissociation constants of the reaction were similar to those of the adult receptor, about 10^{-10} M (Table 1).

The distribution, however, was different. In the adult the

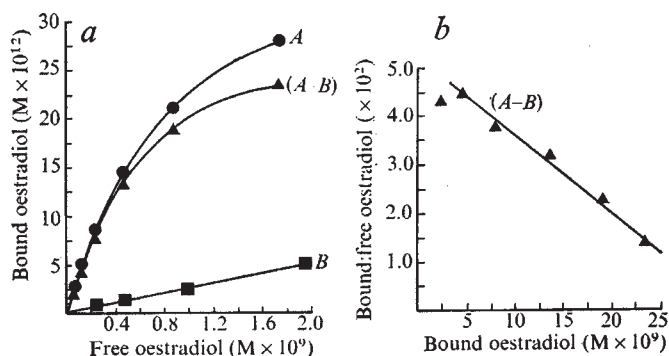


Fig. 1 *a*, The binding of ³H-oestradiol to cytosol from brain of 5-d-old rats; cytosol was from a female in this example. Cytosol (110,000g × 1 h; phosphate buffer, pH 7.3) samples were incubated with various concentrations (2 × 10⁻⁹ M to 2 × 10⁻¹⁰ M) of 2, 4, 6, 7 ³H-oestradiol-17 β (100 Ci mmol⁻¹) for 30 min at 30° C. Control incubations contained, in addition, an excess (10⁻⁷ M) of unlabelled DES. 0.2 ml fractions were placed on small columns (0.45 × 10 cm) of Sephadex LH-20 in buffer, maintained at 0–4° C. Samples were washed with 0.2 ml buffer and allowed to remain in contact with the gel for 150 min before they were eluted (0.7 ml), and bound radioactivity estimated. *A*, total binding; *B*, binding in the presence of excess DES; (*A-B*) DES suppressible binding, obtained by subtraction. *b*, Scatchard representation of *A-B*. We detected no DES suppressible binding in either blood or heart cytosol.

receptors are most concentrated in hypothalamus and amygdala and are relatively sparse in cortex. In the brain of the neonatal animal equal amounts of high affinity oestrogen binding material were found in both HA and C and in both sexes (Table 1); concentrations were similar to those of the receptor in the adult female hypothalamus. The binding material is, at least in part, protein: incubation with pronase, but not with DNase or RNase, destroyed its ability to bind oestradiol. Binding activity was reduced after incubation with protamine sulphate or with *p*-hydroxymercuribenzoate. Thus the protein is similar to the adult receptor in that it is acidic, and the

Table 1 Distribution and dissociation constants of reaction (K_d) of DES suppressible, high affinity binding of ^3H -oestradiol-17 β , in cytosol from brain of 5-d-old rats

	Abundance of oestradiol binding sites Sites g^{-1} wet weight ($\times 10^{-10}$)			Reaction dissociation constant $K_d \times 10^{10}$ (M)		
	Hypothalamus	Amygdala	Cortex	Hypothalamus	Amygdala	Cortex
Adult female	19.8 $\pm$ 1.2	8.3 $\pm$ 0.4	1.8 $\pm$ 0.3	1.5 $\pm$ 0.3	1.08 $\pm$ 0.16	1.7 $\pm$ 0.6
5-d-old female	11.8 $\pm$ 1.8		13.4 $\pm$ 1.1	6.1 $\pm$ 1.2		6.4 $\pm$ 1.5
5-d-old male	10.7 $\pm$ 1.0		15.9 $\pm$ 1.9	5.7 $\pm$ 0.6		6.3 $\pm$ 1.0

Data are compared with values for binding in cytosols from brain of adult female rats¹⁵. Parameters were calculated from Scatchard representation of binding isotherms. Avogadro's number was used in the calculation of the number of binding sites. Values represent means $\pm$ s.e.m. of at least five observations.

Table 2 The specificity of the binding reaction in cytosol from brains of 5-d-old rats compared with that of the receptor of the adult female hypothalamus

Competitor	Order of K_d	5-d-old					
		Adult ♀ H	♀ HA	♀ C	♂ HA	♂ C	
Ethamoxxytriphetol	—(MER 25)	10 ⁻⁶ M	~1	~1	~1	~1	~1
5 α -Androstan-3 α , 17 β -diol	—(3 α diol)	10 ⁻⁷ M	3.4 $\pm$ 0.6	4.8 $\pm$ 1.3	5.9 $\pm$ 2.3	3.4 $\pm$ 1.2	~10
5 α -Androstan-3 β , 17 β -diol	—(3 β diol)	10 ⁻⁸ M	3.3 $\pm$ 0.4	6.4 $\pm$ 1.9	9.1 $\pm$ 3.4	7.9 $\pm$ 1.1	6.0 $\pm$ 2.6
<i>trans</i> Clomiphene	—(TCI)	10 ⁻⁸ M	8.0 $\pm$ 1.5	11.4 $\pm$ 6.1	10.1 $\pm$ 3.3	14.4 $\pm$ 3.7	13.4 $\pm$ 2.5
<i>cis</i> Clomiphene	—(<i>cis</i> Cl)	10 ⁻⁸ M	1.3 $\pm$ 0.5	0.62 $\pm$ 0.22	4.6	4.1 $\pm$ 2.4	1.0 $\pm$ 0.17
Oestriol	—(E ₃)	10 ⁻⁹ M	1.7 $\pm$ 0.1	1.2 $\pm$ 0.5	2.5 $\pm$ 1.5	2.8 $\pm$ 1.0	5.3 $\pm$ 3.6
17 α -Oestradiol	—(17 α)	10 ⁻⁹ M	2.2 $\pm$ 0.2	3.4 $\pm$ 1.4	3.2 $\pm$ 0.8	5.5 $\pm$ 2.3	3.7 $\pm$ 1.7
Oestrone	—(E ₁)	10 ⁻⁹ M	1.7 $\pm$ 0.1	31 $\pm$ 7	22 $\pm$ 4	12 $\pm$ 4	29 $\pm$ 14
16-Epi oestriol	—(Epi) E ₃	10 ⁻⁹ M	1.3 $\pm$ 0.1	2.0 $\pm$ 0.6	3.9 $\pm$ 1.7	0.8 $\pm$ 0.2	3.4 $\pm$ 1.9
11 β -Methoxy-17 α -ethynyl- 17 β -oestradiol	—(Ru 2858)	10 ⁻¹⁰ M	7.1 $\pm$ 0.7	6.0 $\pm$ 1.5	4.1 $\pm$ 1.1	5.2 $\pm$ 1.6	5.6 $\pm$ 1.4
17 β -Oestradiol	—(17 β)	10 ⁻¹⁰ M	1.2 $\pm$ 0.1	5.7 $\pm$ 1.7	5.5 $\pm$ 1.0	3.2 $\pm$ 0.6	3.7 $\pm$ 1.1
DES	—	10 ⁻¹¹ M	6.0 $\pm$ 1.0	8.5 $\pm$ 0.9	11.3 $\pm$ 3.1	9.8 $\pm$ 0.3	6.4 $\pm$ 1.1

Dissociation constants of reaction were calculated from the suppression in the presence of unlabelled test substances of binding of ^3H -oestradiol in the conditions specified in the legend to Fig. 1. Where standard errors are quoted the data are the mean values of four observations. Testosterone, 5 α -dihydrotestosterone, progesterone, corticosterone and cyproterone (10⁻⁶ M) had no effect on the binding of ^3H -oestradiol.

integrity of -SH groups is important for the binding reaction.

The specificity of the reaction is the same in both areas and in both sexes and is broadly similar to the adult receptor (Table 2): potent oestrogens have high, and non-oestrogenic steroids have low binding affinities.

The low affinity for MER 25 of both the oestradiol receptor of adult hypothalamus and the oestrogen binding material from neonatal brain is somewhat surprising; however, MER 25 is an effective antioestrogen only when administered in high doses¹⁶ and it protects the brain against masculinisation only when given some time before exposure to androgen⁵.

The affinities of the protein in brain cytosol from 5-d-old rats for oestradiol-17 β and for oestrone are apparently lower than those of the adult (Table 2): but these differences may be artefacts due to the presence of brain EBP in the incubation medium.

The high binding affinities of the synthetic oestrogens DES and Ru 2858 are particularly interesting. Both compounds have a low affinity for blood EBP (ref. 13) and both affect the developing brain in a manner similar to that of oestradiol. Ru 2858, at least, is very much more potent than oestradiol-17 β in its effects on the developing brain (C. Doughty, unpublished).

We propose that the high affinity, oestrogen-specific binding protein of neonatal brain cytosol is a receptor which is involved in the sexual differentiation of the central nervous system. Recent autoradiographic work¹² supports this view. After injection of ^3H -oestradiol into newborn female rats radioactivity was concentrated in the nuclei of cells in the hypothalamus and amygdala: uptake was inhibited by concurrent administration of oestradiol or testosterone, but not by unlabelled 5 α -dihydrotestosterone, which cannot be converted to oestrogen *in vivo*.

The role of the putative cytoplasmic receptor of the neonatal cortex has yet to be determined. As aromatising enzymes are absent in the cortex it is unlikely to be involved in the normal

process of masculinisation but may be involved in a, so far undefined, organising effect of oestradiol itself.

This work was supported by the Medical Research Council.

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Received August 2, 1974.

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